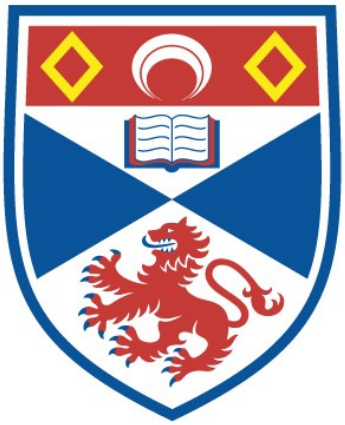
DNA DOUBLE-STRAND BREAKS MEASURED BY THE NON-DENATURING FILTER ELUTION TECHNIQUE FOLLOWING X-RAY AND RESTRICTION ENDONUCLEASE TREATMENT

Nina D. Costa

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Thesis submitted for the degree of PhD in Radiation Biophysics
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Declaration

I, Nina D. Costa, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any degree or professional qualification.

Signed N. Costa Date 27/3/90

I was admitted to the faculty of Science of the University of St. Andrews under Ordinance General No. 12 on 4/88 and as a candidate for the degree of Ph.D. on 9/89

Signed N. Costa Date 27/3/90

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of PhD.

Signature of the Supervisor Date
Dr P.E. Bryant

27/3/90

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ABBREVIATIONS:

AT	-	ataxia telangiectasia
ara A	-	9-β-D-arabinofuranosyladenine
ara C	-	1-β-D-arabinofuranosylcytosine
Bam H1	-	restriction endonuclease from *Bacillus amyloliquefaciens* H; recognising and cleaving the sequence G GATCC
bp	-	base pairs
Bq	-	becquerel; unit of radioactivity = 1 disintergration/s
BSA	-	bovine serum albumin
CA	-	chromosomal aberrations
CHEF	-	clamped homogeneous electrical field (gel electrophoresis)
CHO	-	Chinese hamster ovary cell line
^{14}C-TdR	-	^{14}C-labelled thymidine; 2-^{14}C thymidine
ds	-	double-stranded (DNA)
dsb	-	DNA double-strand break/s
EAT	-	Ehrlich ascites tumour cell line
Eco R1	-	restriction endonuclease from *Escherichia coli* RY 13; recognising and cleaving the sequence G AATTC
EDTA	-	ethylenediamine tetra-acetic acid
HBSS	-	Hanks buffered salts solution
HEPES	-	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
H_2O_2	-	hydrogen peroxide
^{3}H-TdR	-	tritium labelled thymidine; [*Me* -^{3}H]thymidine
LET	-	linear energy transfer
LMDS	-	locally multiply damaged sites
MEM	-	mimimal essential medium (Eagles)

NLS	-	sodium-N-lauroylsarcosine or N-dodecanoyl-N-methylglycine
-OH	-	hydroxyl group
PBS	-	phosphate buffered saline
PCC	-	premature chromosome condensation
Pvu II	-	restriction endonuclease from *Proteus vulgaris* ; recognising and cleaving the sequence CAG CTG
RE	-	restriction endonuclease
SDS	-	sodium dodecyl sulphate or sodium lauroyl sulphate
SB	-	storage buffer
SPB	-	sodium phosphate buffer
ss	-	single-stranded (DNA)
ssb	-	DNA single-strand break/s
TCA	-	trichoracetic acid
TPAH	-	tetrapropylammonium hydroxide
tris	-	tris(hydroxymethyl)methylamine
V79	-	Chinese hamster lung fibroblast cell line
xrs	-	X-ray-sensitive mutant of Chinese hamster ovary cell line

ABSTRACT

The non-denaturing filter elution technique of Bradley and Kohn (1979) is widely used as an assay for DNA double-strand breaks (dsb) in mammalian cells. Results characteristically obtained with this assay following exposure of cells to ionising radiation, namely that of non-linear induction of dsb and rapid biphasic repair, are not in agreement with those of the neutral velocity sedimentation technique where the biophysical basis of measurement is understood. The discrepancy in the results obtained with these two techniques with regard to both induction and repair of dsb has led to a controversy, in particular concerning the manner in which the data obtained with the neutral elution technique should be interpreted (Ahnström's Comment on Radford 1985; Hutchinson 1989). The aim of this project was therefore to attempt to test the assumed specificity of the non-denaturing filter elution technique as an assay for dsb.

Optimization of the lysis and eluting conditions was followed by detailed X-ray dose-response and repair experiments with the CHO K1 cell line. A comparative study was performed using the xrs 5 cell line, a radiosensitive mutant of the CHO K1 line chosen for its characteristic marked deficiency in dsb repair, yet normal ability to rejoin single-strand breaks (Kemp *et al.* 1984; Costa and Bryant 1988).

Previous reports by Bryant and Blöcher (1982), and Iliakis and Bryant (1983) revealed that the DNA synthesis inhibitors ara A and ara C strongly inhibit dsb repair as assayed by neutral velocity sedimentation. I thus adopted an experimental strategy in which the effect of ara A and ara C on putative dsb repair was examined using the non-denaturing filter elution assay. Only limited inhibition of dsb repair by these nucleoside analogues was observed with the non-denaturing filter elution technique in contrast to the complete inhibition of dsb repair as measured by neutral velocity sedimentation for the same concentrations of DNA synthesis inhibitor (Bryant and Blöcher 1982; Iliakis and Bryant 1983). These results suggest that the two above mentioned techniques are detecting disparate types of dsb, as manifested by the differential requirement of the repair mechanism of these breaks for DNA polymerization.

A further approach was the introduction of restriction endonucleases (RE) into mammalian cells by electroporation, to induce dsb in the absence of other types of lesions. The observed increase in the rate of elution of the DNA of RE-treated cells substantiates the ability of the non-denaturing filter elution assay to detect cellular dsb. Surprisingly *Pvu* II was found to remain active inside the cell for up to 24 h, and the continual incision of the DNA by the enzyme thwarted the possibility of monitoring the repair of these dsb. A noteworthy result was the relative inability of RE which generate cohesive-ended dsb (*e.g. Bam* H1 and *Eco* R1) to induced measurable numbers of dsb as compared with the blunt-end cutter *Pvu* II. A hypothesis of a competition between the induction of dsb by RE and the subsequent repair is offered as explanation, where the repair of cohesive-ended dsb is assumed to take place at a higher rate than that of blunt-ended dsb. A comparative study using the xrs 5 mutant cell line, known to be deficient in dsb repair, revealed enhanced levels of RE-induced dsb which would support the notion that the levels of dsb reflect a competition between RE-incision and dsb repair.

In summary, this study validates the measurement of dsb by the non-denaturing filter elution method and provides new evidence for the mode of induction of dsb by RE which has not been hitherto possible. Finally, the work indicates the way in which cells handle different types of dsb which may be similar to the manner in which the variety of dsb induced by ionising radiation are dealt with.

CHAPTER 1

INTRODUCTION

1.1 Ionising radiation and cell lethality

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1.7 Specific aims of this project

This thesis concerns the widely used but rather controversial non-denaturing filter elution assay for DNA double-strand breaks (dsb). In this introductory chapter, the significance of dsb in relation to the biological effects of ionising radiation is explained. Diversity in the interpretation of the results of dsb studies using the non-denaturing filter elution assay, has led to a controversy and these interpretations are discussed in the light of accepted theories in the field of radiobiology. An experimental strategy is proposed which would test the validity of the non-denaturing filter elution assay to detect DNA double-strand breaks.

1.1 Ionising radiation and cell lethality

Exposure of mammalian cells to ionising radiation *in vivo* or *in vitro* can induce a variety of biological changes in the cells, including cell death or loss of proliferative capacity, chromosomal aberrations, mutations and oncogenic transformations. For cultured cells, cell death is defined as the loss of reproductive integrity, or alternatively cell survival is an assay of the ability of a cell to produce a cell colony. This is known as the 'clonogenic' assay and a plot of cell survival as a function of radiation dose thus yields a survival curve. This was one of the first techniques used to quantitate the deleterious effects of X-rays on cells.

The DNA within the nucleus is now generally accepted to be the main sensitive target for radiation damage and hence responsible for the biological effects of radiation, despite evidence that also implicates the nuclear membrane (Alper 1979).

1.2 The structure of DNA and chromatin

The ability to perceive the biophysical mechanism of induction of damage by ionising radiation and the nature of the subsequent DNA lesions depends on an understanding of the structure and conformation of DNA.

The DNA molecule is a double helix consisting of two sugar-phosphate chains (or phosphodiester strands) on the outside and bases on the inside. The bases (adenine - A, cytosine - C, guanine - G, and thymidine - T) are attached covalently to the sugar of the phosphodiester backbone and form complementary pairs with bases on the opposite strand by means of hydrogen bonds (A=T or G≡C). The sequence of these bases forms the genetic code which is transcribed for protein synthesis, upon which all organic processes depend.

During S-phase of the cell cycle the DNA is duplicated in its entirety such that at cell division each daughter cell will receive a copy of the full genomic content of the parent cell. Each strand acts as a template for the new DNA strand, a process which is mediated by a DNA polymerase enzyme (thought to be polymerase α in mammalian cells). During this replication process the two helically wound strands of DNA need to be separated and the new strands are synthesized in the 5' to 3' direction. Other than DNA polymerase, several other enzymes are required for replication; to effect unwinding at the replication fork (helicase), helix-destabilization, rewinding (topoisomerases) and to join fragments on the lagging strand (ligases). Since the original strands of DNA are conserved as one of the strands of the newly synthesized DNA, this mechanism of DNA replication is said to be semi-conservative.

Unless replication is taking place, the DNA is condensed into the chromatin structure consisting of several orders of packing. In Fig. 1.1 a

schematic diagram of current models of levels of organisation in the chromatin illustrates how condensation of the long DNA strand is possible. During interphase the chromatin structure is thought to be comprised of the 30 nm fibre which is attached at regular intervals, by means of proteins, to the nuclear lamina and internal matrix to form the so-called DNA loops. Futhermore, two general classes of chromatin have been distinguished on the basis of their appearance under the microscope (Alberts *et al.* 1983): condensed or heterochromatin which is transcriptionally inactive and less condensed euchromatin which is actively transcribed. Just prior to cell division, superpacking of the DNA is in effect achieved when the chromatin of interphase condenses to form the chromosomes visible during metaphase of mitosis. Clearly the extent of condensation of the DNA into chromatin changes markedly during the cell cycle; it changes from the relaxed structure of actively transcribed euchromatin or the open structure of the replication forks during S-phase, to the highly condensed chromosomes at metaphase of mitosis.

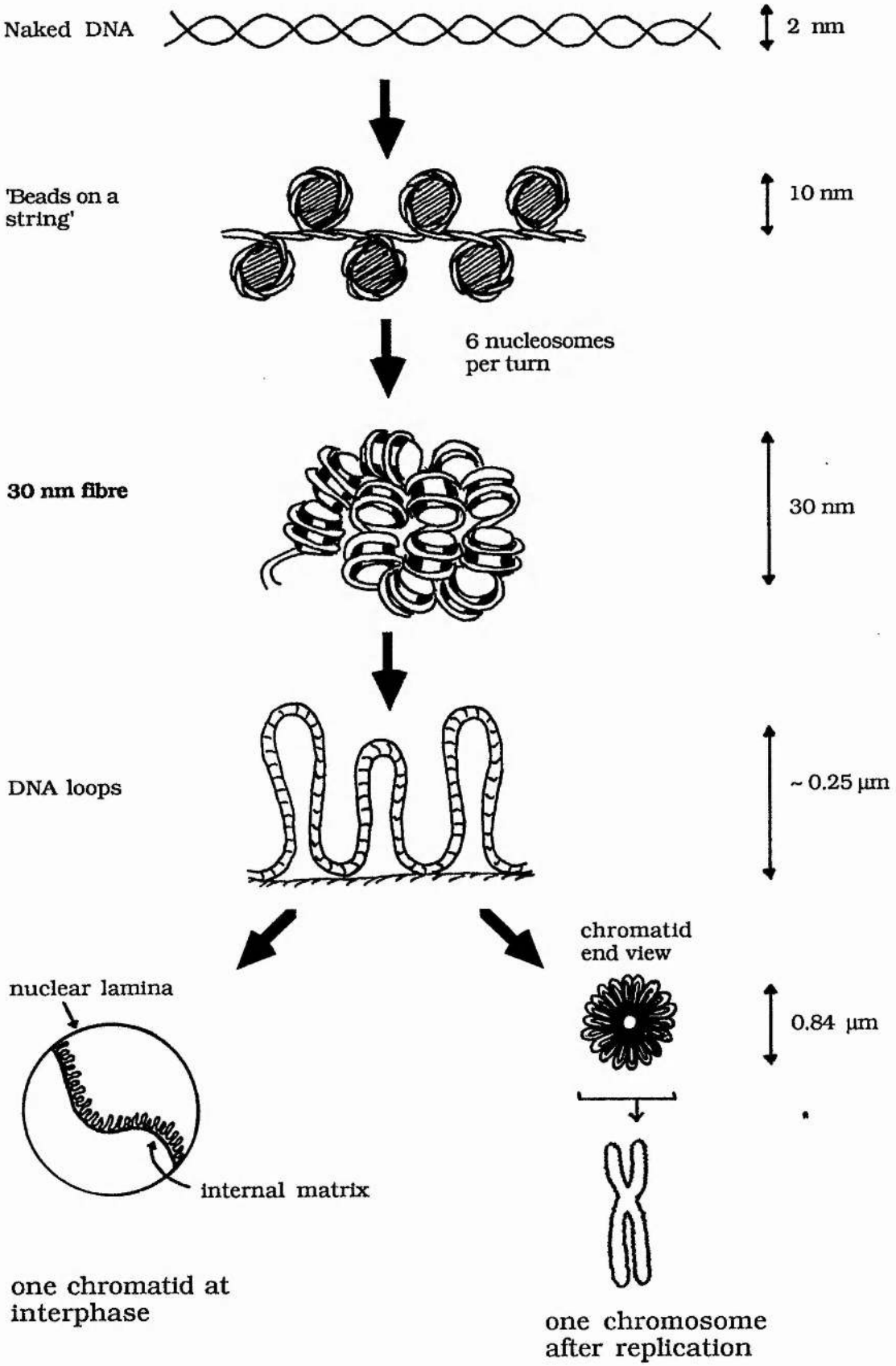

Figure 1.1 Schematic diagram of current models of DNA superpacking into chromatin taken from Pienta and Coffey (1984)

1.3 Radiation-induced DNA damage

In attempts to describe the physical interaction of ionising radiation with DNA it is not known what effect the higher-order structure of the DNA within the chromatin will have. The DNA molecule is therefore often considered as simply the 2 nm double helix surrounded by associated proteins and ions.

1.3.1 *DNA lesions*

Ionising radiations such as X-rays, γ-rays, α-particles or neutrons interact with cellular material in a way that produces tracks of secondary electrons within the cell. The high local energy imparted by these ionising particles or electrons (Goodhead and Brenner 1983) can either be absorbed directly by the DNA molecule (so-called direct action) or can cause the formation of chemical radicals which may attack the DNA (indirect action). The absorption of radiation energy within the DNA leads to the formation of various types of lesions *viz.* DNA strand breaks, base damage, crosslinks and LMDS - locally multiply damaged sites (Ward 1985; Téoule 1987).

DNA strand breaks can be divided into 2 categories; single-strand breaks (ssb; break in just one of the phosphodiester strands), or double-strand breaks (dsb) where two strand breaks occur in close proximity on opposing strands. The frequency of ssb and base damage induction are thought to be similar (~1100/Gy/genome) whereas dsb are 20-30 times less frequent (~40/Gy/genome) (*e.g.* Blöcher 1982; van der Schans *et al.* 1982).

Crosslinks between one DNA strand and associated proteins or between the two DNA strands are possible. The induction frequency of these crosslinks is relatively low; 133/Gy/genome for DNA-protein

crosslinks and even lower for interstrand-crosslinks (1st L.H. Gray Workshop) and these lesions are therefore probably of minor importance when compared to strand breaks or base damage, in contributing to the biological effects of ionising radiation. DNA crosslinks may however be lethal by interfering with DNA separation during S-phase.

Mammalian cells have the capacity to repair a considerable amount of damage to the genome, as illustrated by the fact that an absorbed dose of 1 Gy typically leads to an average of somewhat less than 1 'lethal lesion' per cell. Base damage or loss of a base is thought to be rectified by excision of a few nucleotides in the damaged region, the gap is then filled by DNA polymerase and finally the nick in the strand is sealed by ligase (Painter and Young 1972; Fox and Fox 1973). Base damage is therefore in effect converted to ssb, and hence the polymerization and ligation steps are common to both repair mechanisms. The different types of DNA strand breaks are not equally important in cell killing and it has been postulated that ssb and therefore also base damage are not responsible for cell lethality (Fox and Fox 1973; Hesselwood 1978; Ward *et al.* 1985; Prise *et al.* 1989b). The main body of evidence points to double-strand breaks (dsb) and other LMDS as the most critical lesions (Hutchinson 1974; Ho 1975; Resnick and Martin 1976; Frankenberg *et al.* 1984; Resnick 1978; Hutchinson 1974; Blöcher and Pohlit 1982; van der Schans *et al.* 1982; Radford 1986; Ward 1985; 1986).

Work based on radiation sensitive strains of yeast *e.g.* rad 52, which cannot repair dsb, shows that 1-2 dsb constitute a lethal event (Ho 1975; Resnick and Martin 1976; Frankenberg *et al.* 1984). This evidence gave rise to the hypothesis that **dsb** are the lesions within the DNA which could potentially result in mammalian cell lethality (*e.g.* Radford 1986a; 1986b). This hypothesis has been supported by studies on mutants of mammalian cell lines that are hypersensitive to ionising radiation and

which exhibit diminished levels of dsb repair (Kemp *et al.* 1984; Evans *et al.* 1987; Wlodek and Hittelman 1987; Costa and Bryant 1988; Zdzienicka *et al.* 1988). It is however important to note that the extent of repair of dsb may not be the only determinant of cell death, as illustrated by numerous studies on cell lines which differ in their radiosensitivity but not in their ability to repair DNA lesions (Lehmann and Stevens 1977; Rahmsdorf *et al.* 1981; Koval and Kazmar 1988a; Swiegert *et al.* 1989; Peacock *et al.* 1989). There is strong evidence that the fidelity of repair also plays a crucial role, shown by studies on cells from individuals with the autosomal recessive genetic disease ataxia telangiectasia (AT). These studies attributed the inherent radiosensitivity of the AT cells to inaccuracies in dsb rejoining rather than to lack of repair (Cox *et al.* 1984; 1986; Debenham *et al.* 1987). It is reasonably self-evident that the genomic position of the unrepaired or misrepaired dsb will affect the biological outcome, *i.e.* a lesion in the extensive, non-vital regions of the genome might not challenge the ability of the cell to survive to the same extent as a lesion within a gene which controls a vital cellular function (unless it leads to terminal deletion of a chromosome fragment which may itself contain both useful and non-essential sequences).

Despite the lack of direct evidence most investigators agree that unrepaired or misrepaired dsb play a crucial role in lethality of mammalian cells (Thacker and Stretch 1985; Bryant 1988). By employing restriction endonucleases (RE) that generate dsb to model radiation damage, it has been shown that there is a strong correlation between dsb and the induction of chromosomal aberrations (Bryant 1984; Obe *et al.* 1985), cell lethality (Bryant 1985), mutations (Obe *et al.* 1986), and oncogenic transformation (Bryant and Riches 1990). RE are therefore powerful tools with which to investigate the role of dsb, in the absence of the other lesions which are always present in cells exposed to

ionising radiation. Yet it must be borne in mind that the dsb induced by radiation and RE differ in one major aspect, namely that of the nature of the end groups of the strand breaks: RE breaks have 'clean' 3'-hydroxyl and 5'-phosphoryl termini, whereas radiation induced breaks are likely to have 'dirty' ends and the structure of these breaks is likely to determine the way in which the cell will deal with the lesion (Bryant 1988; Ward 1986; Ward *et al.* 1987).

1.3.2 *Correlation of cytogenetic damage with DNA double-strand breaks*

The nanometer dimensions of the DNA molecule excludes the possibility of visualising the breaks within the strands, even with electron microscopy. The deleterious effects of ionising radiation can however be visualised in a light microscope as abnormalities in the metaphase chromosomes or in prematurely condensed chromosomes (PCC) as fragments.

Chromosomal aberrations (CA) comprise various abnormalities which include gaps, deletions (breaks) and exchanges. CA are thought to result from dsb which are not repaired, resulting in deletions, and dsb which are misrepaired, yielding exchanges (Bender *et al.* 1974). The dsb origin of CA is implicated by a large body of indirect evidence: For example, enzymes such as *Neurospora* single strand endonuclease which convert ssb in the DNA of X-irradiated cells to dsb, have been shown to cause approximate doubling in the dsb frequency and a corresponding increase in CA (Natarajan *et al.* 1980). Inhibitors of dsb repair have been shown to enhance the yield of X-ray induced CA (Iliakis *et al.* 1988b; Mozdarani and Bryant 1987; 1989), while dsb repair deficient mutants have shown higher levels of CA than the wild-type parent lines following X-ray exposure (Kemp and Jeggo 1986; Darroudi and Natarajan 1987a; 1987b) or RE treatment (Bryant *et al.* 1987).

In experiments in which the time between X-irradiation and mitosis was varied to encompass G_2 phase cells, the half-time of the rate of disappearance of deletions is similar to that of dsb rejoining as measured by neutral velocity sedimentation, which has lead to a speculative correlation between the deletions and dsb (Mozdarani and Bryant 1987). In the same article the increased frequency of exchanges in the presence of the dsb repair inhibitor ara A was attributed to misrepair events in dsb rejoining. Using the PCC technique in cells treated with ^{125}I, Iliakis *et al.* (1987) measured similar repair kinetics for PCC fragments as that of dsb repair as measured by neutral elution after X-ray exposure.

The above evidence points to unrepaired or misrepaired dsb as the origin of CA, but the frequencies of CA are numerically very much lower than the frequencies of dsb present in the DNA. For this reason a comparison of the results of cytogenetic and dsb assays should be conducted with caution.

1.4 Models of DNA double-strand break (dsb) induction and repair

1.4.1 *Double-strand break induction*

Biophysical studies in which the radiation track structure is compared with the biological effects reveal that the probability of tracks overlapping is negligible even at high doses of ten to hundreds of grays. This evidence has led to the theory that dsb are produced predominantly by individual radiation tracks (Goodhead 1989). The breaks in opposing DNA strands could both be due to the direct action of localised clusters of ionisations within the track end of a single electron track of sparsely

ionising radiations such as X- or γ-rays (Goodhead and Brenner 1983), or formed in conjunction with protein or water molecules which are closely associated with the DNA (indirect action). Indirect action is thought to occur by a mechanism by which highly reactive species such as hydroxyl radicals, a major radiolysis product of water, react with DNA to give rise to strand breaks and other types of lesions. High LET or densely ionising radiation, such as neutrons or α-particles, is on the other hand more likely to generate dsb by local massive destruction (Tobias *et al.* 1980; Coquerelle *et al.* 1987; Goodhead 1989). Most biophysical models assume random induction of damage by ionising radiation throughout the DNA within the nucleus due to the random nature of energy deposition of radiation. Numerous authors have however shown that certain regions in the DNA, *viz.* transcriptionally active DNA, might be preferentially broken (Chui *et al.* 1982; Warters *et al.* 1987; Heussen *et al.* 1987). The main concept that needs to be emphasised is that dsb are thought to be the result of a single track event, which would imply that the number of dsb induced should increase linearly with the dose of ionising radiation.

Ionising radiation is known to induce dsb with various end structures *e.g.* Von Sonntag *et al.* (1981) found evidence of DNA strand breaks with (a) phosphate groups at both the 3' and 5' terminals with the sugar and the base missing, (b) strand breaks with an altered sugar on the 3' terminal and 5'-phosphate end group and (c) alkali-labile sites *i.e.* alterations to the sugar moeity and loss of the base such that the strands would split under alkali conditions. Clearly, breaks with 5'-phosphoryl and 3'-hydroxyl ends, commonly referred to as 'clean' breaks, could easily be rejoined by simple ligation. In comparison 'dirty' breaks which carry a damaged sugar or base, or gaps in the strands resulting from destruction of the deoxyribose would require several enzymes to be repaired *e.g.* exonuclease for the removal of damaged termini, polymerase to fill in the

gap and ligase to reseal the nick in the strand (Landbeck and Hagen 1973; Henner *et al.* 1982). Von Sonntag *et al.* (1981) were essentially referring to ssb induced by low LET or sparsely ionising radiation but the 'dirty' nature of the termini is likely to be similar for dsb.

It is also obvious that two ssb in close proximity on opposite strands could behave as a dsb, but this situation as mentioned before, is only probable at extremely high doses of radiation or for free DNA irradiated in aqueous solution where high concentrations of hydroxyl radicals are induced. It is not unreasonable to assume that base damage, which is converted to ssb by endonuclease action, in close proximity to a ssb could result in the formation of a dsb (Natarajan *et al.* 1980; Ahnström and Bryant 1982; Bryant 1986). The possibility therefore that non-lethal lesions can interact to give rise to a potentially lethal lesion (*i.e.* a dsb) cannot be excluded.

1.4.2 *Repair of double-strand breaks*

It has been suggested that there may be differences in the repair or misrepair of dsb induced by sparsely or densely ionising radiations, but current evidence in the literature is not conclusive on this point. Van der Schans *et al.* (1983) and Maki *et al.* (1986) found no apparent difference in the rate or extent of repair, while others found a diminished rate of rejoining and more residual unrepaired breaks following exposure of cells to high LET radiation (Ritter *et al.* 1977; Roots *et al.* 1979; Bryant and Blöcher 1980; Coquerelle *et al.* 1987; Blöcher 1988; Fox and McNally 1988). The extent of misrepair has not been elucidated for high LET radiation although it is known that higher frequencies of exchange aberrations occur after high LET exposure (Edwards *et al.* 1980). Ward (1985) has suggested that locally multiply damaged sites (LMDS) would

present problems for accuracy of cellular repair mechanism due to the lack of template integrity.

The biochemical mechanisms of dsb repair are as yet unknown, although indirect evidence has been sought to reveal the nature of the repair *e.g.* through the use of chemical agents which interrupt repair or the use of bacterial mutants deficient in a specific repair pathway (Collins 1987). There is evidence that the repair of dsb in bacteria requires recombination with an intact region of a homologous DNA molecule (Krasin and Hutchinson 1977; Weibezahn and Coquerelle 1981; Picksley *et al.* 1984; Lopez and Coppey 1987). The rad 52 mutant of yeast which is unable to repair dsb is also known to be recombination defective (Resnick 1975). Resnick (1976) and Szostak *et al.* (1983) have proposed models of dsb repair involving recombination, which basically requires normally occurring degradative and repair enzymes and a heteroduplex recombinant structure. According to these models, the recombination event is initiated by degradation of the ends of the dsb by exonucleases to form large single-stranded regions or gaps of 20-30 bases. These are then filled in by using large DNA segments from a homologous chromosome in a reciprocal or non-reciprocal fashion. DNA polymerase is subsequently required to synthesise the lacking complementary sequences in both chromosomes and ligase acitivity is necessary to complete the rejoining process.

The dsb repair mechanism in mammalian cells is however not known. Resnick and Moore (1979) detected recombination in S/G_2-phase cells but not in G_0 cells and thus concluded that recombination was not associated with dsb repair on account of the occurrence of dsb repair in all phases of the cell cycle. Moore *et al.* (1986) found the X-ray-sensitive mutant of the CHO cell line, xrs 5, known to be deficient in dsb repair (Kemp *et al.* 1984), to have a reduced ability to carry out

homologous recombination in an *in vitro* system, the extent of which parallelled its deficiency in dsb repair. The possibility of a recombination mechanism of dsb repair in mammalian cells seems likely as this uses the integrity of the homologous DNA information to ensure accuracy of rejoining in the absence of a template in the opposing strand (Debenham *et al.* 1987).

Chemical and enzymatic end group studies have identified a range of dsb termini induced by ionising radiation (as mentioned above in section 1.4.1) and thus repair of ionising radiation-induced dsb would probably call upon a range of enzymatic processes from simple ligation to recombination. Based on the fast dsb repair kinetics measured by neutral elution, Radford (1987a) proposed that simple ligation could represent the mechanism of dsb repair. The enzyme responsible for ligation, ligase, requires a 3'-OH and 5'-phosphoryl substrate and therefore exonuclease cleaning of the ends of 'dirty' dsb would be a prerequisite for a ligation mechanism of dsb rejoining (Lennartz *et al.* 1975). Such a repair process would seem feasible, but it could possibly lead to the deletion of base pairs which might have a deleterious effect on the integrity of the genome. On the other hand it could be possible that mammalian cells have more than one system for dsb rejoining; one involving emergency ligation (which is preferable to allowing the dsb to remain open for a considerable period) and a second recombination mechanism which would ensure accurate repair (Weibezahn and Coquerelle 1981). It might be possible that incorrectly ligated dsb are, at some later stage in the cell cycle, detected and the integrity of the sequence restored by recombination. Meuth (1990) has in fact found evidence of a proof-reading exonuclease in the replication complex of CHO cells.

Recent recombinant DNA techniques have allowed the assessment of the fidelity of repair (Thacker 1986) and AT cells which are proficient

in dsb repair (Lehmann and Stevens 1977; Coquerelle and Weibezahn 1981; Thierry *et al.* 1985) have been shown to have a high level of dsb that are misrepaired (Cox *et al.* 1984; 1986). It has been suggested that this misrepair process is the cause of the hypersensitivity to ionising radiation characteristic of the AT cells (Cox *et al.* 1984; 1986). The low fidelity of dsb repair in AT cells has been attributed to large deletions or rearrangements at the site of the dsb as a result of exonuclease digestion of the termini before ligation. On the basis of this data Cox *et al.* (1986) postulated the existence of a competition between ligation and exonuclease digestion of the ends of the broken DNA, which is shifted towards degradation in AT cells. Further evidence then led Debenham *et al.* (1987) to speculate that this could be due to the action of topoisomerase II, which might protect the dsb from degradation in normal cells by binding to the end structures, but that this interaction might be defective in AT cells.

1.5 Techniques used to assay for dsb in mammalian cells

In this section, the biochemical techniques that have been used to measure the induction and repair of DNA dsb in mammalian cells are described and the results characteristically obtained with these methods are discussed. Unlike cytogenetic or recombinant DNA assays, these techniques measure the cumulative (bulk) effect of radiation on a large number of cells (in the order of 1.10^4 - 1.10^6 cells/sample). Caution needs to be adopted when comparing the results of different assays if non-isogenic cell types have been used. Chromatin structure can differ markedly between cell lines and this could determine the response of

cells to ionising radiation (*e.g.* Wheeler and Wierowski 1983; Koval and Kazmar 1988a).

The neutral velocity sedimentation technique (Lehmann and Stevens 1977; Blöcher 1982) is, at present, the only assay that enables direct measurement of the molecular weight of the DNA (based on the distance sedimented by the DNA in a neutral sucrose gradient under centrifugation) and thus of the actual numbers of dsb present in the DNA. The technique unfortunately has the disadvantages of being extremely time consuming and not particularly sensitive *i.e.* the doses used are generally outside the 'biologically relevant' range. Using this technique, several authors have found that dsb are formed as a linear function of dose in bacteria (Krasin and Hutchinson 1977), yeast (Resnick and Martin 1976; Frankenberg-Schwager *et al.* 1979) and in mammalian cells (Corry and Cole 1973; Lehmann and Stevens 1977; Blöcher 1982; Ahnström and Bryant 1982). The fact that neutral velocity sedimentation studies have found dsb to be linearly dependent on dose (except at very high doses of ~4000 Gy where ssb are so frequent that they by chance interact to form a dsb) represents strong evidence in favour of the 'single-track' theory of dsb induction referred to earlier. The neutral velocity sedimentation technique is therefore generally regarded as the most reliable assay for dsb.

The DNA unwinding technique employs alkali lysis conditions (pH 12) which causes the DNA strands to separate and unwind from points of breakage. Under carefully controlled lysis conditions the extent of unwinding is proportional to the number of strand breaks. This method therefore detects a total number of breaks (*i.e.* ssb plus dsb), but Bryant and Blöcher (1980) devised an experimental protocol by which DNA unwinding can be used to follow the kinetics of dsb repair. In this respect they found good similarity between repair kinetics as measured

by the DNA unwinding and neutral velocity sedimentation techniques. The DNA unwinding technique has the advantage of its technical simplicity as well as good reproducibility.

Both the above techniques detected first-order repair kinetics *i.e.* an exponential decrease in the number of dsb remaining with time. The half-times ($t_{1/2}$) of repair were estimated at ~3.5 h (Lehmann and Stevens 1977) and t_{37} = 2-4 h depending on culture conditions (Bryant and Blöcher 1982; Blöcher and Pohlit 1982). These results thus implied a single, relatively slow repair component which in turn signified that there may only be one mechanism of dsb repair. Using the DNA synthesis inhibitor ara A (9-β-D-arabinofuranosyladenine) Bryant and Blöcher (1982) showed that this repair mechanism had an absolute requirement for DNA polymerization. This would support the notion of a recombination mechanism of dsb repair as against ligation.

Another rapid and simple method for detecting DNA breaks is the DNA precipitation assay of Olive (1988). This technique is based on the differential precipitibility of varying DNA fragment sizes (large fragments precipitate while the smaller fragments remain in the supernatant), but a major drawback of this technique is its insensitivity for the detection of dsb. Under the neutral conditions used to assay for dsb the percentage of DNA precipitated decreases linearly as a function of X-ray dose, indicating a linear increase of dsb with dose albeit at relatively large X-ray doses (40 - 200 Gy). No dsb repair studies have yet been undertaken using this precipitation technique.

Recently Blöcher *et al.* (1989) showed that the newly developed CHEF (clamped homogeneous electrical field) gel electrophoresis technique was a suitable method for measuring dsb in mammalian DNA, that is based on the movement of negatively charged DNA molecules in an electric field. This assay exhibits good sensitivity and allows the

detection of dsb down to ~2 Gy of X-rays. In their initial report Blöcher *et al.* (1989) observed non-linear induction of dsb with dose, but in a subsequent article (Blöcher 1990) he explains that this may be due to an artefact of the technique, *viz.* the extraction properties of the DNA molecules out of the well, and once corrected for the assay gave linear induction of dsb. Stamato and Denko (1990) using the asymmetric field inversion gel electrophoresis (AFIGE) technique have also reported a linear induction of dsb with dose of ionising radiation. The dsb repair rate as measured by CHEF electrophoresis is considerably faster than that measured by the neutral velocity sedimentation or DNA unwinding, with a repair time constant $t_{1/2}$ of 30-40 min (Blöcher *et al.* 1989). A kinetic study revealed biphasic rather than first-order repair kinetics, but this has as yet not been repeated by other investigators.

The non-denaturing filter elution technique of Bradley and Kohn (1979) is currently the most widely used assay for DNA dsb and this technique exhibits non-linear dsb induction and biphasic repair kinetics with a $t_{1/2}$ of 30-40 min (Kemp *et al.* 1984). This technique and these results are discussed in greater detail in the following section (1.6).

The above techniques are quantitative assays of the induction frequency of dsb and the extent of dsb repair that cannot comment on the type of repair effected or whether different types of DNA termini are more prone to erroneous repair. Recombinant DNA techniques are required to investigate these aspects of the fidelity of dsb repair (Thacker 1986).

It is fairly obvious that the results of studies of induction and repair of dsb depend largely on the assay that was employed. This leaves one to question the accuracy and/or validity of each assay and the possible interpretation of the results, especially in the case of the non-denaturing filter elution assay.

1.6 The controversy surrounding the non-denaturing filter elution technique

In the filter elution technique the filters are thought to act mechanically to impede the passage of the strands of DNA and hence its assumed that the greater the number of breaks present in the DNA the greater the chance of eluting the DNA fragments from the filter. This elution technique was initially used under alkaline conditions (pH 12) in order to discriminate sizes of single-stranded fragments of DNA and therefore to detect DNA ssb (Kohn and Grimeg-Ewig 1973). Variations of this technique have been used to detect alkali-labile sites, DNA-protein crosslinks and DNA interstrand-crosslinks, based on the influence on elution of the extent of protein adsorption to the filter (Kohn 1986; Radford 1986b).

Bradley and Kohn (1979) first used the filter elution assay under conditions where protein adsorption to the filter was minimised and at the lower pH values of 9.6 and 7.4, in order to elute double-stranded DNA and therefore to detect dsb. They showed empirically that neutral filter elution was a sensitive assay for dsb in mammalian cells and found that more DNA eluted from the filter at the higher pH value of 9.6 than at 7.4. Strictly speaking the term 'neutral' is misleading since the technique is usually performed at the alkali pH of 9.6, but the term was coined by Bradley and Kohn to distinguish the assay from the then existing alkaline elution technique (performed at pH 12.2) and to emphasise that at the pH values of 7.4 or 9.6 the DNA retains its double-stranded structure. The technique is therefore also known as non-denaturing filter elution. Throughout this thesis the assay will be referred to as non-denaturing filter elution or simply neutral elution.

The use of non-denaturing filter elution as an assay for DNA dsb has increased dramatically in the past few years due to its technical simplicity and high sensitivity relative to other available techniques, even though absolute quantitation of dsb by this assay is not possible. The technique can be calibrated using cells treated with ^{125}I, however this has proved to be problematical (see Radford 1988 and Hutchinson 1989). The high sensitivity of this technique allows measurements to be made at radiation doses within the survival curve range, thus enabling possible correlations to be established and tested between cell killing and DNA dsb induction and repair.

1.6.1 *The mechanism of non-denaturing filter elution*

The purpose of the fairly rigorous lysis treatment employed by Bradley and Kohn (1979) was to release the DNA from the cells and to remove all DNA-bound proteins and RNA in order to attain unencumbered, free DNA on the filter. They showed that by treating the cell lysate with the restriction endonuclease (RE) *HpA* I, which generates blunt-ended dsb, a considerable increase in the rate of DNA elution was observed. This experiment is usually held to be the strongest piece of evidence that the neutral elution technique measures dsb.

In contrast to the alkaline elution assay, the elution profiles or semi-logarithmic plots of the DNA retention versus elution time, were not straight under non-denaturing conditions, but concave (Bradley and Kohn 1979). These concave elution profiles reflect a fast initial rate of elution, followed by a slower rate at longer elution times. Obviously, and as noted by Bradley and Kohn (1979) the concave elution profiles indicate that portions of the DNA elute with different kinetics. A model which can quantitatively describe this elution behaviour of the large double-stranded DNA fragments from the filter is still however lacking.

A 'spaghetti-in-a-sieve' (*i.e.* double-stranded spaghetti) analogy can be envisaged to conceptualise the mechanism of the technique, whereby during flushing the sieve with water the smaller fragments of spaghetti would stand a greater chance of getting through the holes in the sieve leaving the longer strands behind. The fact that a filter with 2 μm pores can retain DNA with a 1000-fold smaller diameter, implies that the DNA fragments might be of considerable length. This would not be an unreasonable assumption when it is considered that the maximum length of DNA per chromosome is approximately 5 cm. Depending on the distance separating the pores, it is therefore likely that the two ends of any one fragment could attempt to go though two different pores. This idea had been suggested by Nicolini *et al.* (1983) and was incorporated into their model of elution, but it did not fully explain the shape of the elution profiles. Balbi *et al.* (1986) proposed a model in which they considered the physico-chemical properties of the DNA (*i.e.* length and flexibility/superpacking) and the geometric and hydrodynamic configuration of the apparatus, but even this detailed model was of limited success in describing the elution profiles. These models did however emphasise that the elution, although determined by the fragment length of the DNA, is also affected by the flexibility or rigidity of the strands and by the degree of random coiling of the DNA.

Futhermore, a lack of understanding exists regarding the chemical basis of the lysis step of the non-denaturing filter elution technique and this has lead to disagreement among investigators on how to interpret the elution data, a point which is illustrated by the contradicting views regarding the optimum pH of the neutral elution assay. Bradley and Kohn (1979) and Radford (1988) argued that a pH of 9.6 should be used, as this pH would effect more efficient removal of the DNA-binding proteins that could otherwise mask the dsb. Tilby *et al.* (1984) and Evans *et al.* (1986),

on the other hand, argued that at pH 9.6 alkali-labile sites are detected that would not be present at normal intracellular pH and which do not contribute to cell lethality. This issue is discussed in greater detail in chapter 2. It should be noted that high pH during lysis has also been used in the sedimentation (Blöcher 1982) and gel electrophoresis (Schwartz and Cantor 1984) techniques for measuring the induction of dsb.

The controversy surrounding the interpretation of the dose-response and repair kinetic data obtained with the neutral elution assay (considered in the following sections 1.6.2 and 1.6.3) is also in part due to the lack of understanding of the biophysical mechanism of the elution behaviour of the double-stranded DNA.

1.6.2 *The non-linear dose-response relationship for neutral elution*

The non-denaturing filter elution technique measures non-linear induction of dsb with X-ray dose in mammalian cells (Ross and Bradley 1981; Woods 1981; Radford 1985; Sigdestad *et al.* 1987; Wlodek and Hittelman 1987; Prise *et al.* 1987; Swiegert *et al.* 1988; Okayasu *et al.* 1988), in contrast to the linear induction measured by neutral velocity sedimentation. Although it must also borne in mind that the shoulder of the DNA elution dose-response occurs in the low dose region *e.g.* 0-10 Gy, which is below the detection limit (~20 Gy) of the neutral velocity sedimentation technique. These non-linear dose-response curves can either be considered as quadratic (Blazek *et al.* 1989) or as linear-quadratic, *i.e.* a linear increase with a concave shoulder in the low dose region (Okayasu and Iliakis 1989). If the non-linear induction of dsb is not an artefact of the technique, it implies that a significant proportion of individual ionising tracks co-operate to form a dsb. In fact Blazek *et al.* (1989) have proposed, on the basis of the neutral elution assay, that the

induction of dsb by γ-irradiation is proportional to the square of the dose and thus that dsb are entirely due to 'two-hit' events.

Radford's explanation for the shoulder in the dsb induction curve, as measured by neutral elution, was that mammalian cells contain a saturable process for chemical 'shielding' of the DNA from radical attack (Radford 1988). Earlier Radford (1987b) had reported that nuclei showed a near-linear dose-response when irradiated in buffered saline solution, but that addition of sulphydryl compounds restored the shoulder. This data led him to postulate that sulphydryl compounds were responsible for this chemical 'shielding' of cellular DNA and thus for the shoulder at low doses observed in the induction curves. However, the finding of Okayasu and Iliakis (1989) that they could reduce or in fact eliminate the shoulder by appropriate choice of the lysis conditions rather than changing the cellular environment, would refute Radford's explanation of the non-linear dose-response relationship.

Radford (1986a) had also suggested that the shouldered dose-response for neutral elution mirrors the shoulder on the cell survival curves, with the implication that the level of induced dsb reflects the extent of cell killing. This hypothesis is based on extensive studies of; one cell type under a variety of environmental conditions (Radford 1985), cells of different intrinsic radiosensitivities (Radford 1986a), cells synchronised in different phases of the cell cycle (Radford and Broadhurst 1986) and of cells of different ploidy (Radford and Hodgson 1987). This hypothesis has been treated with suspicion since it implies that the intrinsic radiosensitivity of a cell line is determined by an enhanced dsb dose-response rather than its ability to repair dsb, but has nevertheless gained support from other groups (Prise *et al.* 1987; Wlodek and Hittelman 1987; Kelland *et al.* 1988; Peacock *et al.* 1989). The idea that radiosensitive strains of wild-type cell lines show enhanced induction

of dsb is difficult to reconcile with the physics of energy deposition, but could possibly be explained in terms of differences in chromatin structure or in sulphydryl or glutathione content (Radford 1986a).

Numerous groups on the other hand, have published evidence which refutes Radford's hypothesis *e.g.* Iliakis *et al.* (1988a), Iliakis and Okayasu (1988), Wlodek and Hittelman (1988a), Okayasu *et al.* (1988), Wheeler *et al.* (1988), Swiegert *et al.* (1988) and Swiegert *et al.* (1989). In two articles by Okayasu and colleagues (Okayasu *et al.* 1988; Okayasu and Iliakis 1989), the shouldered dose-response curve was attributed to incomplete separation of the DNA from the chromatin during lysis, and to variations in the organization of DNA through the cell cycle. Iliakis *et al.* (1988a) could find no difference in the dose-response curves, and therefore in the induction of dsb, of the wild-type CHO K1 and the xrs 5 (X-ray-sensitive) mutant cell lines, despite a 20-30 fold difference in their level of radiosensitivity. Wlodek and Hittelman (1988a), Wheeler *et al.* (1988) and Swiegert *et al.* (1988) could also find no correlation between the level of induced dsb detectable by neutral elution and cell survival in cell lines of different radiosensitivities. Another important finding that opposed Radford's hypothesis was that of Okayasu and Iliakis (1989), who obtained a <u>linear</u> elution dose-response, after selection of appropriate lysis conditions in a cell line (CHO) which has a shouldered survival curve.

Okayasu *et al.* (1988) using synchronised CHO cells found that the dose-response of the neutral elution assay fluctuated significantly with phase of the cell cycle at which the cells were irradiated. It was not so much the state of growth that affected the elution (the dose-response of exponential and plateau phase cells were not that different), but rather the progression of the cells into the S-phase of the cell cycle. The elution properties of the DNA showed a cyclic response within S-phase - starting with a maximum at the beginning of S-phase, reaching a minimum in the

middle of S-phase and rising again to the maximum value after the completion of DNA synthesis. These fluctuations could be attributed to either variations in the level of induction of dsb (as per Radford) or to changes in the elution characteristics of the DNA during S-phase and the associated inception of DNA synthesis. Based on the results of Blöcher *et al.* (1983) who found the same level of dsb induction in all the cycle phases using the neutral velocity sedimentation technique, Okayasu *et al.* (1988) proposed that the fluctuations in the DNA elution dose-response during S-phase were due to the changes in DNA structure and chromatin conformation associated with DNA replication. The implication was that the replication fork complexes, present in the middle of S-phase, could somehow withstand disruption during lysis and thus diminished elution was observed. Additionally, Okayasu *et al.* (1988) could find no correlation between the DNA elution dose-response and cell killing in the various phases of the cell cycle, contrary to Radford's data (Radford and Broadhurst 1986).

Basically, the two schools of thought can be reduced to a choice between accepting the non-linear dose-response of the assay as a reflection of the underlying mechanism of lesion induction (*e.g.* Blazek *et al.* 1989; Radford 1987b) or considering it an artefact of the technique (Okayasu *et al.* 1988; Okayasu and Iliakis 1989).

1.6.3 *Biphasic repair kinetics with neutral elution*

The non-denaturing filter elution technique detects a repair kinetic which differs from that measured by neutral velocity sedimentation and this has greatly contributed to the controversy: - neutral velocity sedimentation measures a single slow component of dsb repair with a repair rate constant $t_{1/2}$ of 2-4 h (Bryant and Blöcher 1982; Blöcher and Pohlit 1982), whereas neutral elution studies suggest biphasic repair

kinetics with a considerably faster half-time of dsb disappearance *viz.* 30-40 min (Kemp *et al.* 1984). When comparing the results of these two techniques it should be borne in mind that larger doses and longer incubation times are generally used in neutral velocity sedimentation studies than in neutral elution assays.

The biphasic repair kinetics of the neutral elution assay can be interpreted to reflect two distinct repair components *viz.* an initial component reflecting fast repair ($t_{1/2}$~5 min) followed by a slower repair component with much longer $t_{1/2}$ of 0.5-2 h (*e.g.* Weibezahn and Coquerelle 1981; Radford 1983; 1987; Rowley and Kort 1988; Swiegert *et al.* 1989). It is plausible that the slow component of repair as measured by neutral elution, with a $t_{1/2}$ of 0.5-2 h could be similar to the single repair component of sedimentation studies ($t_{1/2}$=2-4 h). The essence of the controversy however concerns the origin of the rapid component of repair, with a $t_{1/2}$ of 2-10 min, and it has been suggested that this component reflects ssb rather than dsb repair on account of the very rapid kinetics (Ahnström's Comment on Radford 1985; Hutchinson 1989). The experimental reports on this matter are confusing; Van Ankeren and Meyn (1987) found a correlation between the fast component and ssb repair whereas Fox and McNally (1988) could not. At least two studies of cells exposed to hydrogen peroxide, proficient at inducing DNA ssb, would argue against this idea (Bradley and Kohn 1979; Prise *et al.* 1989b). The latter authors only detected dsb at a high concentration of H_2O_2 at which the induction dsb via closely spaced ssb was possible. These experiments do not however exclude the possibility that ssb, when present in conjunction with dsb, could increase the elution by increasing the flexibility of the DNA strands (Ahnström's Comment on Radford 1985) or by making the DNA strands more susceptible to shearing (Hayward 1974). The idea that ssb could affect

DNA elution remains popular since many of the controversial issues surrounding the neutral elution assay are resolved if the fast repair component is considered to reflect ssb rejoining (Hutchinson 1989).

A further discrepancy between the results obtained with the neutral elution and neutral velocity sedimentation assays concerns the issue of the repair rate in the different phases of the cell cycle. Blöcher *et al.* (1983) and Rydberg (1984) reported comparable dsb repair kinetics in all phases of the cell cycle, whereas Radford (1987a) reported that mitotic cells did not exhibit the same kinetics as G_1, S or asynchronous cells as assayed by neutral elution. In contrast to the biphasic dsb repair kinetics of G_1,S or asynchronous cells, mitotic cells repaired with first-order kinetics with a $t_{1/2}$ that exceeded the slow component of the other cells (Radford 1987a). On the basis of these results Radford suggested that the two components of dsb repair were due to differences in the chromatin structure of DNA *i.e.* the dsb in euchromatin might be repaired more rapidly than those in the tightly condensed heterochromatin. This also lead Radford (1987a) to postulate the existence of a single dsb repair mechanism, the rate of which is limited by the accessibility of repair enzymes to the DNA, in contrast to the model of Weibezahn and Coquerelle (1981) in which two repair mechanisms were proposed (*viz.* fast ligation and a slower process, possibly recombination).

1.7 Specific aims of this project

It is clear that the above controversy surrounding the non-denaturing filter elution assay covers many fundamental issues concerning the biophysics of dsb induction and the physico-chemical mechanisms of dsb repair. The aim of this project was therefore an attempt to throw

some light on this problem by taking a closer look at the non-denaturing filter elution technique.

The strategy adopted was firstly to optimise the lysis and eluting conditions of the non-denaturing filter elution assay in order to achieve maximum sensitivity of the assay and good reproducibility in the measurement of dsb induction and repair in mammalian cells after X-ray exposure. The increased sensitivity would allow the repair of dsb to be followed over extended incubation times, *i.e.* up to 8 h post-irradiation, which were comparable with those used in sedimentation or DNA unwinding studies. Detailed dose-response and repair kinetic investigations were undertaken using the Chinese hamster ovary (CHO K1) wild-type cell line and compared with those of a X-ray sensitive mutant strain, xrs 5. The xrs 5 cell line was chosen on account of its characteristic marked deficiency in dsb repair, yet normal ability to rejoin ssb (Kemp *et al.* 1984).

Another approach was to use DNA synthesis inhibitors, known to inhibit dsb repair, in order to determine whether the repair of the lesions detected by neutral elution were inhibited to the same extent as dsb repair measured by neutral velocity sedimentation. In this series of experiments another mammalian cell line *viz.* Ehrlich ascites tumour (EAT) cells was utilised, such that the results could be directly compared with previous investigations of the effect of the same DNA synthesis inhibitors on EAT cells, as assayed by velocity sedimentation and DNA unwinding (Bryant and Blöcher 1982; Iliakis and Bryant 1983).

A third strategy was adopted in which restriction endonucleases (RE) were used to generate dsb in cells and to investigate the ability of the neutral elution assay to detect these dsb and to distinguish between different types of dsb. The RE were introduced into the cells by means of the newly developed electroporation technique, which has proven to be

efficient and more reliable than previously used methods of permeabilization (*e.g.* inactivated Sendai virus). Furthermore various different RE were used to distinguish between elution properties of DNA carrying blunt or cohesive-ended dsb. Lastly, the RE investigation was extended to the xrs 5 cell line to investigate possible differences in the repair of blunt- or cohesive- ended dsb.

Each of the above issues are dealt with in individual chapters, and the significance of the results are discussed at the end of each. In the concluding chapter these results are discussed in the light of current theories concerning the non-denaturing filter elution assay for dsb and finally, all the experimental results are viewed together and possible interpretations thereof are put forward.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

2.2 General materials and methods

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2.2.2 Non-denaturing filter elution

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2.3.1 Radioactive labelling

2.3.2 Composition of the lysis and eluting solutions

2.3.3 Proteolytic enzymes

2.3.4 The effect of pH

2.3.5 Detergents and lysis temperature

2.4 Conclusions

2.1 Introduction

The cell culture and radioactive labelling protocols used throughout this project are described in this chapter. Where individual experiments differed from the general methods described here, the details are provided under the Materials and methods section of the individual chapter.

Most importantly this chapter contains a description of the filter elution technique performed under non-denaturing conditions, the technique which forms the backbone of this project. The methodology followed was basically that of Bradley and Kohn (1979). A literature search soon revealed that most investigators use more or less the same methodology, but that very little attention had been paid to standardising the details of the experimental protocols. The lack of consensus regarding the experimental protocols is possibly due to various investigators who hold differing views as to the factors affecting elution, as mentioned previously in chapter 1 (section 1.6).

Most workers agree that the lysis and eluting conditions should be chosen to achieve DNA that is ideally free of all bound proteins and hence has lost all higher-order chromatin structure, which could otherwise mask DNA damage (Radford 1988). Authors however disagree on how this should be achieved and the experimental protocols under contention are; (a) the pH at which the technique should be performed, (b) the chemical composition of the lysis and eluting solutions, (c) the use of proteolytic enzymes in the lysis solution and (d) the type and concentration of detergent used in the lysis solution and (e) the lysis temperature. (a) to (e) are discussed in greater detail below.

(a) *The effect of pH*

Bradley and Kohn (1979) found that the DNA of cells that had been exposed X-rays or restriction endonucleases exhibited more extensive elution at pH 9.6 than at pH 7.4, and they attributed this to more effective removal of proteins or interfering cellular material from the DNA at the higher pH. This point of view is also held by Radford as explained in his recent review article (Radford 1988). On the other hand Tilby *et al.* (1984) and Evans *et al.* (1986) proposed that the greater elutability at pH 9.6 was due to hydrolysis of alkali-labile bonds in the damaged DNA which would not occur at normal intracellular pH. The recent results of Flick *et al.* (1989) suggest that the additional dsb at pH 9.6 are in fact the product of existing ssb and pH 9.6-labile sites in close proximity on the opposite strands. This issue, regarding the pH at which the non-denaturing filter elution assay should be performed, remains largely unresolved and hence many investigators tend to report their findings at both pH values (*e.g.* Sigdestad *et al.* 1987; Van Ankeren and Meyn 1987; Koval and Kazmar 1988a; Rowley and Kort 1988).

(b) *Composition of lysis and eluting solutions*

As described in the original paper of Bradley and Kohn (1979), identical solutions for the lysis and elution steps were used except for the addition of 0.5 mg/ml proteinase K to the lysis solution just prior to use. This solution consisted of 0.05 mol/l tris, 0.05 mol/l glycine, 0.025 mol/l Na_2EDTA and 0.07 mol/l SDS (2 %) adjusted to either pH 9.6 or 7.4. Tris (tris(hydroxymethyl)methylamine) and glycine were used in a buffering capacity while the Ca^{++} chelating action of EDTA (ethylenediamine tetra-acetic acid) inhibited the action of nucleases which would otherwise degrade the DNA. SDS (sodium dodecyl sulphate or sodium lauryl

sulphate), a strong detergent, was used to lyse the cells and to free the DNA of proteins.

Shortly afterwards Ross and Bradley (1981) reported using a lysis solution of above mentioned composition but substituting the eluting solution with a buffer containing 0.06 mol/l TPAH (tetrapropylammonium hydroxide) and 2 % SDS. Woods (1981) had used similar lysis conditions but an elution buffer of 0.02 mol/l EDTA (free acid form) and sufficient TPAH to give a pH of 9.6. Subsequent studies (*e.g.* Kohn *et al.* 1980; Zwelling *et al.* 1981; Iliakis and Okayasu 1988) have used various combinations of the above reagents in the eluting solution. Other than in a study by Koval and Kazmar (1988b), not much attention has been given to these variations in the composition of the eluting solution. They in fact found that the choice of eluting solution (tris versus TPAH) did affect the results obtained with the neutral elution assay at both pH values.

(c) *Use of proteolytic enzymes*

Proteinase K, the enzyme most widely used in the neutral elution assay is isolated from the fungus *Tritirachium album* Limber (Ebeling *et al.* 1974) and is known to have a broad and powerful proteolytic action which frees DNA of RNA, proteins and degrading enzymes (Gross-Bellard *et al.* 1973). Proteinase K was found to have a pH-optimum in the range of 7.5-12.0 and to be very active in the presence of the detergent SDS and metal-chelating EDTA (Gross-Bellard *et al.* 1973), which makes it an ideal proteolytic enzyme for use in the non-denaturing filter elution technique.

Pronase is a proteolytic enzyme that had been used in the rigorous isolation of DNA for the neutral velocity sedimentation technique (Blöcher 1982) and hence was also investigated. Pronase is isolated from *Streptomyces narensis* and shows maximum activity at pH 7.5, but has been shown to be inactivated by EDTA (Hiramatsu 1967).

Proteolytic enzymes like proteinase K or pronase, are added to the lysis solution just prior to use to avoid loss of activity due to self-degradation and are included to effect the removal of all DNA bound proteins. Bradley and Kohn (1979) reported that the presence of proteinase K in the lysis solution significantly increased the elution of the DNA from the filter and therefore addition of proteinase K was adopted as routine practice in the neutral elution assay. Recently Swiegert *et al.* (1988) and Kuo *et al.* (1987) however reported evidence to the contrary *i.e.* they found the presence of proteinase K to have no effect on the elution.

(d) *Detergents and lysis temperature*

The detergent most commonly used in the non-denaturing filter elution assay is SDS (sodium lauryl sulphate or sodium dodecyl sulphate). SDS is an anionic detergent that denatures cell membranes and splits DNA-protein complexes but does not interact with nucleic acids because of its negative charge (Noll and Stutz 1968). It is also known to reduce nuclease activity (Marmur 1961) and to impede the adsorption of proteins to hydrophobic surfaces such as the polycarbonate membrane (Kohn *et al.* 1980). The SDS concentration of 0.07 mol/l (2%) generally used in the lysis solution, represents a 20-fold higher concentration than employed to disrupt protein associations and to maintain proteins in a denatured state for polyacrylimide gel electrophoresis (Weber and Osborne 1969). It is therefore thought unlikely that any proteins remain bound to the DNA at this concentration of detergent (Swiegert *et al.* 1988).

In contrast, Okayasu *et al.* (1988) attributed the characteristic curvilinear dose-response curve of the non-denaturing filter elution technique to the incomplete removal of the DNA-bound proteins by SDS

and proteinase K. Okayasu and Iliakis (1989) subsequently reported obtaining linear dose-response curves, implying more effective removal of the proteins, by employing the stronger lysis conditions of the use of NLS (sodium-N-lauryl sarcosine) rather than SDS in conjunction with an increase in the temperature of lysis treatment from 20 °C (room temperature) to 60 °C.

NLS, an amide derivative of SDS, is also an anionic detergent and although not much information is available on the action of NLS, as far as can be gauged it is similar to that of SDS (Evans and Gurd 1972; Helenius and Simons 1975). NLS has the additional advantage of remaining soluble under storage conditions at 4 °C and at a concentration of 0.07 mol/l, whereas 2 % SDS precipitates out of solution at this temperature.

In view of the lack of consensus regarding the experimental protocols and the often opposing results obtained by different authors, it was deemed necessary to undertake a series of preliminary experiments in order to select a standard procedure to be used in the experiments reported in this thesis. The range of testing was more or less limited to existing protocols as the aim of this project was to investigate the 'state of the art' non-denaturing filter elution technique.

2.2 General materials and methods

2.2.1 *Cell culture, radioactive labelling and irradiation*

Chinese hamster ovary cells (line CHO K1) were routinely grown in Eagle's minimal essential medium (MEM) supplemented with 10 % v/v calf serum to which 100 µmol/l $FeCl_3$ had been added. The addition of $FeCl_3$ was necessary to saturate the high levels of transferrin present in

calf serum (MacLoed *et al.* 1990). Asynchronous populations of exponentially growing cells were used throughout. 75 cm^2 plastic tissue culture flasks (Sterilin) were seeded with 1.10^6 cells and labelled with 3.7 kBq/ml tritiated thymidine (1.59 TBq/mmol). 1 µmol/l of unlabelled thymidine was added to facilitate uniform uptake of the radioactive thymidine (^{3}H-TdR). After the flasks had been gassed with 5 % CO_2 and sealed, they were incubated at 37 °C for 48 hours.

After trypsinization (0.5 g/l trypsin and 0.2 g/l EDTA) cells were resuspended in MEM to a cell concentration of $1.5\text{-}3.10^6$ /ml. 3 ml aliquots of the cell suspension were placed in 5 ml plastic, bijou bottles and gassed with 5% CO_2 before sealing. The samples were then placed on ice and allowed to equilibrate to 4 °C before irradiation.

Cells were exposed to X-rays (250 kV_p, 14 mA and 0.5 mm Cu filtration) at 4 °C and at a dose-rate of 5.8 Gy/min. Doses were checked by ferrous sulphate dosimetry (Frankenberg 1969). After irradiation the cells were returned to ice and kept at 4 °C for the neutral elution step.

2.2.2 *Non-denaturing filter elution*

The neutral elution apparatus consisted of 10 reservoirs (50 ml syringe barrels) each attached to a Swinnex filter holder (Millipore), which could support a ø=25 mm polycarbonate filter. A low speed peristaltic pump with 10 sampling lines, was adjusted to give a constant flow-rate of 2-3 ml/h.

Polycarbonate filters (Nucleopore) with 2 µm pore size were used throughout. 2 µm is the most widely used pore size in the filter elution technique even though Kohn (1986) found that varying the pore size between 0.45-5 µm had little or no effect on the elution kinetics of X-irradiated cells. Polycarbonate filters were used since they have a low capacity for protein adsorption (Kohn *et al.* 1980).

In general (the details are discussed later in this chapter), the cell samples were diluted in approximately 10 ml ice-cold phosphate buffered saline (PBS), poured into the reservoirs and allowed to drip through under gravity, and in so doing loading the cells onto the polycarbonate filters. The number of cells per filter was standardised at the relatively low number of 5.10^5/filter to ensure that the DNA strands of various sizes behaved independently (Bradley and Kohn 1979), although my preliminary data showed that twice that number *i.e.* 1.10^6 cells per filter did not adversely affect the elution properties of irradiated DNA. A further 10 ml of ice-cold PBS was added, just before the previous volume had dripped through, to rinse the cells. Once the second volume of PBS had flowed out under gravity, the Swinnex holders were removed and 1 ml of the lysis solution (composition to be discussed later in this chapter) was gently pipetted into the space above the filter. After 1 h at room temperature or 60 °C the Swinnex holders, which had been stoppered to prevent evaporation of the lysis solution, were reattached to the funnels. 40 ml of eluting buffer (composition to be discussed later in this chapter) was poured into the reservoirs and the elution initiated with the pumps set at a flow-rate of 2-3 ml/h. In most experiments the volume resulting from the first hour of elution (3 ml) was collected as the first fraction, followed by the collection of 3 hourly fractions over 15-17 h.

10 ml Optiphase MP (LKB) was added to each of the fractions, which were vortexed and the radioactivity per sample determined by liquid scintillation counting in a LKB Rackbeta scintillation counter. The polycarbonate filters were removed from the Swinnex holders and thoroughly vortexed in 5 ml Filter Count (Packard) before counting.

'Elution profiles' were obtained by calculating the fraction of radioactivity that remained on the filter at the end of each collection time. The first fraction, which contained lysis debris and unincorporated

radioactive label was not included in this calculation. Assuming that the DNA had taken up the ^{3}H-TdR in a uniform manner, the fraction of radioactivity retained reflects the fraction of DNA retained. The fraction of DNA eluted was thus obtained using the formula:

$$[1-f_I]-[1-f_C]$$

where f_I = fraction of radioactivity retained after 16 h of elution in the treated (e.g. X-irradiated) cell sample and

f_C = fraction of radioactivity retained after 16 h of elution in the untreated cell sample.

2.3 Results and discussion of preliminary non-denaturing filter elution experiments

2.3.1 *Radioactive labelling*

Radioactive labelling of the DNA with ^{3}H-TdR or ^{14}C-TdR (tritiated or ^{14}C-thymidine) was necessary for the detection and quantitation of the DNA in each eluted fraction. Cycling CHO cells were used which readily incorporate thymidine and therefore the radioactive label into the newly synthesised DNA during S-phase of the cell cycle. However, once the label has been taken up the DNA is exposed to the internal β-rays emitted during the decay of either ^{3}H or ^{14}C, which could result in DNA damage (*e.g.* Dikomey and Franzke 1986). ^{14}C was found to be less damaging to the DNA per disintergration than ^{3}H by virtue of the different ranges of the emitted β-particles (Jorgensen *et al.* 1987). An optimisation was therefore sought between sufficient radioactive label to ensure good scintillation counting statistics for each fraction and a level of labelling that would not increase the background level of elution *i.e.* the fraction eluted in the untreated (or control) cells. CHO cells were incubated for

48 h in the presence of various amounts of radioactive thymidine (^{3}H-TdR and ^{14}C-TdR) and the results, measured by filter elution at pH 9.6, are shown in Table 2.1.

Radioactivity (kBq/ml)	Fraction of ^{3}H-activity eluted	Fraction of ^{14}C-activity eluted
1.85	0.08 +/- 0.01	0.08 +/- 0.01
3.70	0.06 +/- 0.00	0.06 +/- 0.01
7.40	0.05 +/- 0.01	0.05 +/- 0.00
11.10	0.04 +/- 0.01	0.05 +/- 0.01

Table 2.1 The fraction of radioactivity eluted (at pH 9.6) for CHO cells that had been incubated in the presence of increasing amounts of radioactive thymidine for 48 h.

The outcome was rather surprising in that an increase in the amount of radioactive thymidine, for both ^{3}H-TdR and ^{14}C-TdR, caused a decrease in background elution level (*i.e.* the fraction of untreated DNA eluted). A possible explanation for this tendency is that with increasing amounts of radioactivity, added as label, the contribution of the background counts (counts registered in the absence of a radioactive source) to the total counts measured per fraction is diminished. Thus, relative to the amount of radioactivity detected on the filter, the amount of radioactivity eluted (total counts per fraction) would seem to decrease with increasing amounts of radioactive label.

^{14}C-labelling was not shown to be advantageous over ^{3}H-labelling and since a labelling concentration of 3.7 kBq/ml gave sufficiently high counts in each fraction, it was decided to standardise on labelling the seeded flasks (at 1.10^{6} CHO cells) with 3.7 kBq/ml ^{3}H-TdR or ^{14}C-TdR for 48 h.

2.3.2 *Composition of the lysis and eluting solutions*

The pH of the lysis and eluting solutions corresponded in all neutral elution experiments *e.g.* when the technique was performed at pH 9.6 both the lysis and eluting solutions were adjusted to that pH and similarly for pH 7.4. Fig. 2.1 shows the elution kinetics of unirradiated and X-irradiated (to a dose of 20 Gy) CHO cells at pH 9.6. The cells were lysed for 1 h at room temperature in a solution containing 0.05 mol/l tris, 0.05 mol/l glycine, 0.025 mol/l Na_2EDTA, 2 % SDS and 0.5 mg/ml proteinase K. Elution of the DNA was performed using either a tris solution (*viz.* the lysis solution without the proteinase K) or a TPAH buffer (0.02 mol/l EDTA, free acid and ~0.06 mol/l TPAH).

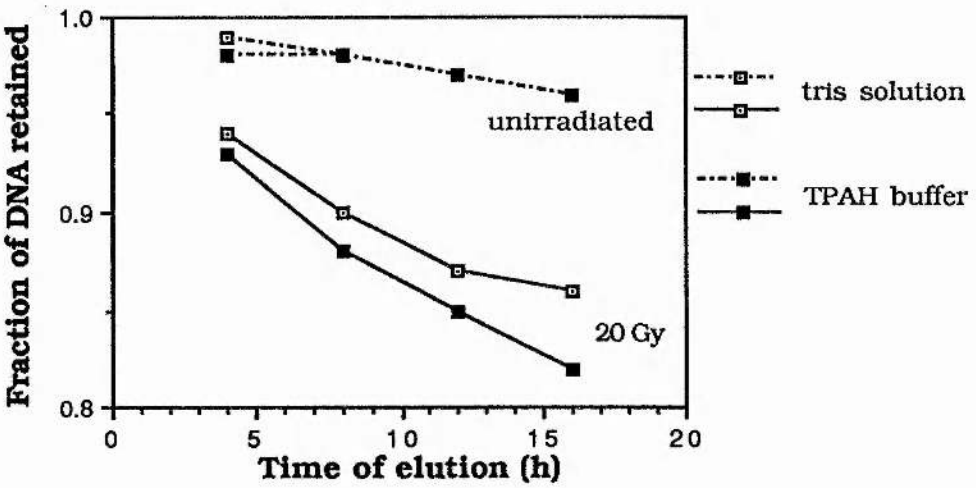


Figure 2.1 The neutral elution profiles (pH 9.6) of unirradiated and X-irradiated (20 Gy) CHO cells eluted with a tris solution or a TPAH buffer, following lysis at room temperature for 1 h.

There is clearly little difference between the elution profiles of the unirradiated cells, the so-called background elution profiles. The TPAH elution buffer resulted in greater elution of the X-irradiated DNA (*i.e.* less DNA retained on the filter) than the tris solution, without drastically altering the shape of the profile. It was therefore decided to use the TPAH elution buffer in all subsequent experiments.

2.3.3 *Proteolytic enzymes*

Most investigators use 0.5 mg/ml proteinase K, freshly dissolved, in a lysis solution containing 0.05 mol/l tris, 0.05 mol/l glycine and 0.025 mol/l Na_2EDTA. In the following experiment, the effect of doubling the proteinase K concentration in a lysis solution containing 0.07 mmol/l SDS (0.002%) and of increasing the SDS concentration 1000-fold to 0.07 mol/l (2%) was compared (Fig. 2.2). Unirradiated and X-irradiated (60 Gy) CHO cells were lysed for 1 h at 20 °C.

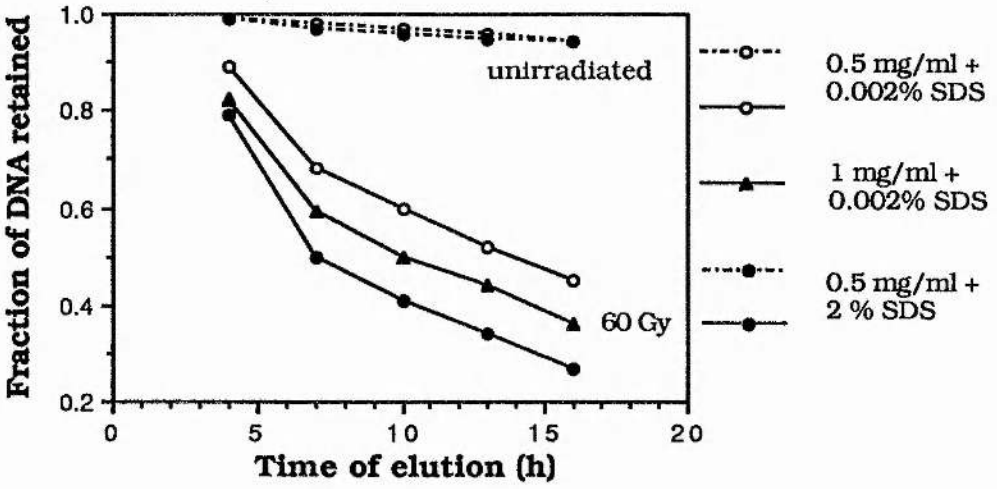


Figure 2.2 The neutral elution (pH 9.6) profiles comparing the effect of various concentrations of proteinase K in conjunction with various SDS concentrations in the lysis solution. Unirradiated and X-irradiated (60 Gy) CHO cells were lysed for 1 hour at 20 °C.

Clearly the higher concentration of proteinase K in the lysis solution (containing 0.002 % SDS) improved the extent of DNA elution for the X-irradiated CHO cells (60 Gy) at pH 9.6, but the best result was obtained when 0.5 mg/ml proteinase K was used in conjunction with the higher SDS concentration of 0.07 mol/l (2 %). Fig. 2.2 also shows a marked improvement in the elution of the DNA when the SDS concentration in the lysis solution was increased to 2 %, without a concomitant increase in

the background elution value. This finding is in disagreement Iliakis and Okayasu (1988), who suggested a reduction in the SDS concentration in the lysis solution from 2 % to 0.002 % since they found that this did not affect the elution properties of the DNA in CHO cells but did significantly reduce the background elution values (in the unirradiated samples). The only obvious difference in the method of Iliakis and Okayasu (1988) and the one described here, was their use of a tris eluting solution rather than the TPAH buffer, but it is not obvious how this could explain the discrepancy in the results presented here with those of the above mentioned authors. The combination of 0.07 mol/l SDS (2 %) and 1 mg/ml proteinase K in the lysis solution was not tested, since optimisation of the lysis conditions in general use was sought. This would ensure that the results obtained in this study were comparable to those of other investigators. It was thus decided to use 0.5 mg/ml proteinase K and 2 % SDS in the lysis solution throughout.

The elution profiles at pH 9.6 of unirradiated and X-irradiated (10 and 50 Gy) CHO cells, after lysis for 1 h at room temperature in the presence of 2 % SDS and equal concentrations of either proteinase K (Sigma) or pronase (CalBiochem), freshly dissolved, are shown in Fig. 2.3.

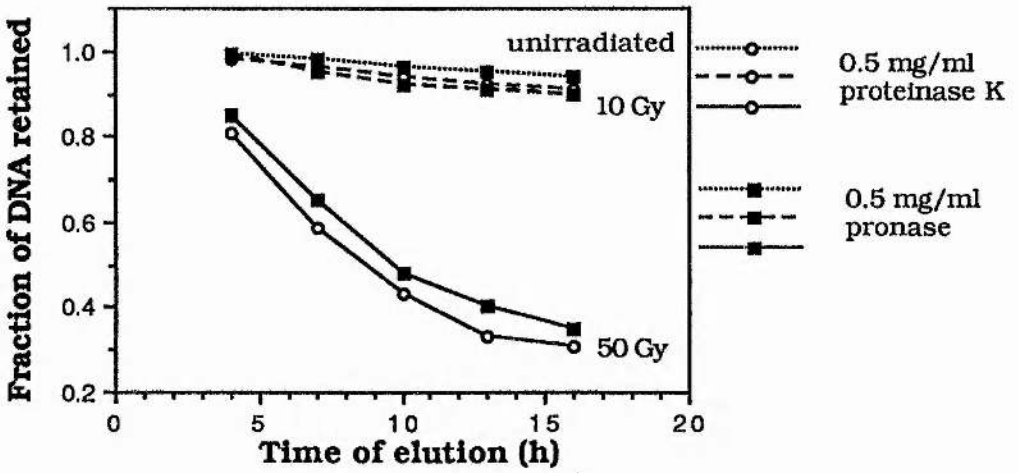


Figure 2.3 The neutral elution (pH 9.6) profiles of unirradiated and X-irradiated (10 and 50 Gy) CHO cells comparing equal concentrations of two proteolytic enzymes, proteinase K and pronase, freshly dissolved in the lysis solution containing 2 % SDS.

The results (Fig. 2.3) show very little difference in the elutability of the DNA of X-irradiated (10 and 50 Gy) cells lysed with either 0.5 mg/ml proteinase K or pronase. As nothing was gained by substituting proteinase K with pronase, it was decided to use proteinase K throughout. Once again, this would ensure comparability with the findings of other investigators.

2.3.4 *The effect of pH*

The elution profiles of X-irradiated CHO cells (20 Gy) at various pH values are shown in Figure 2.4. The cells were lysed for 1 h at 20 °C in lysis solution containing 2 % SDS and freshly dissolved proteinase K at a concentration of 0.5 mg/ml.

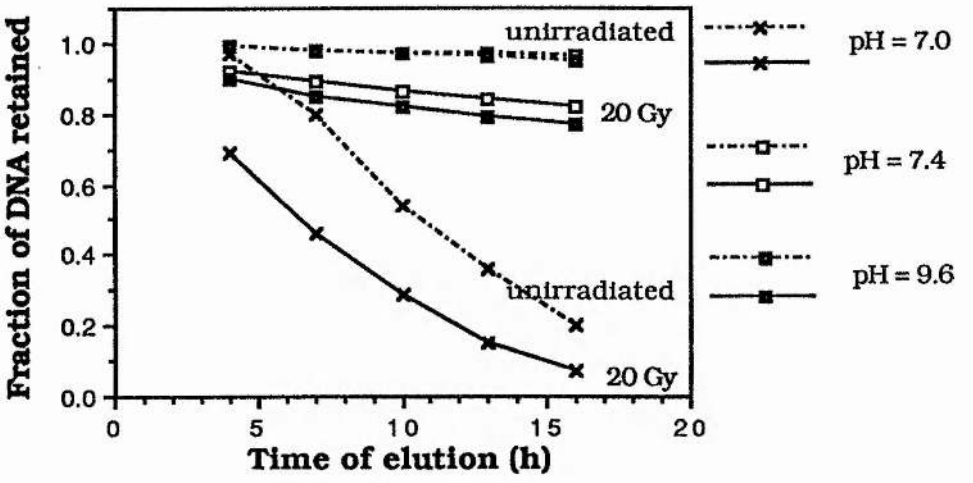


Figure 2.4 The effect of pH on the elution profiles of unirradiated and X-irradiated (20 Gy) CHO cells, lysed for 1 h at 20 °C in the presence of 0.5 mg/l proteinase K and 2 % SDS.

For reasons not understood, the neutral pH of 7.0 caused degradation of the freed DNA evidenced by the increasing fraction of the unirradiated DNA eluted over the 16 h elution period. Once the pH was increased to 7.4 the elution profiles returned to 'normal'. From Fig. 2.4 it can be seen that marginally increased elution of the DNA was achieved at the higher pH of 9.6 without a concomitant effect on controls. The effect of the pH on the results obtained with the neutral elution assay is discussed in greater detail in Chapter 3.

2.3.5 *Detergents and lysis temperature*

The effect of various detergents on the elution kinetics of DNA at pH 7.4 and 9.6 was investigated in conjunction with elevated lysis temperature, since these two factors were found to be closely linked.

Okayasu and Iliakis (1989) had reported an improved (increased) elution rate by replacing SDS in the lysis solution with the detergent NLS, and by increasing the lysis temperature from room temperature (20 °C) to 60 °C. It was thought necessary to verify these major alterations in the

lysis treatment. The resulting elution profiles, at both pH 7.4 and 9.6, of CHO cells exposed to 20 Gy X-rays and lysed for 1 h in the presence of either SDS (Sigma Chemicals) or NLS (Sigma Chemicals) at 20 °C are shown in Fig. 2.5 (a) and after lysis at 60 °C in Fig. 2.5 (b).

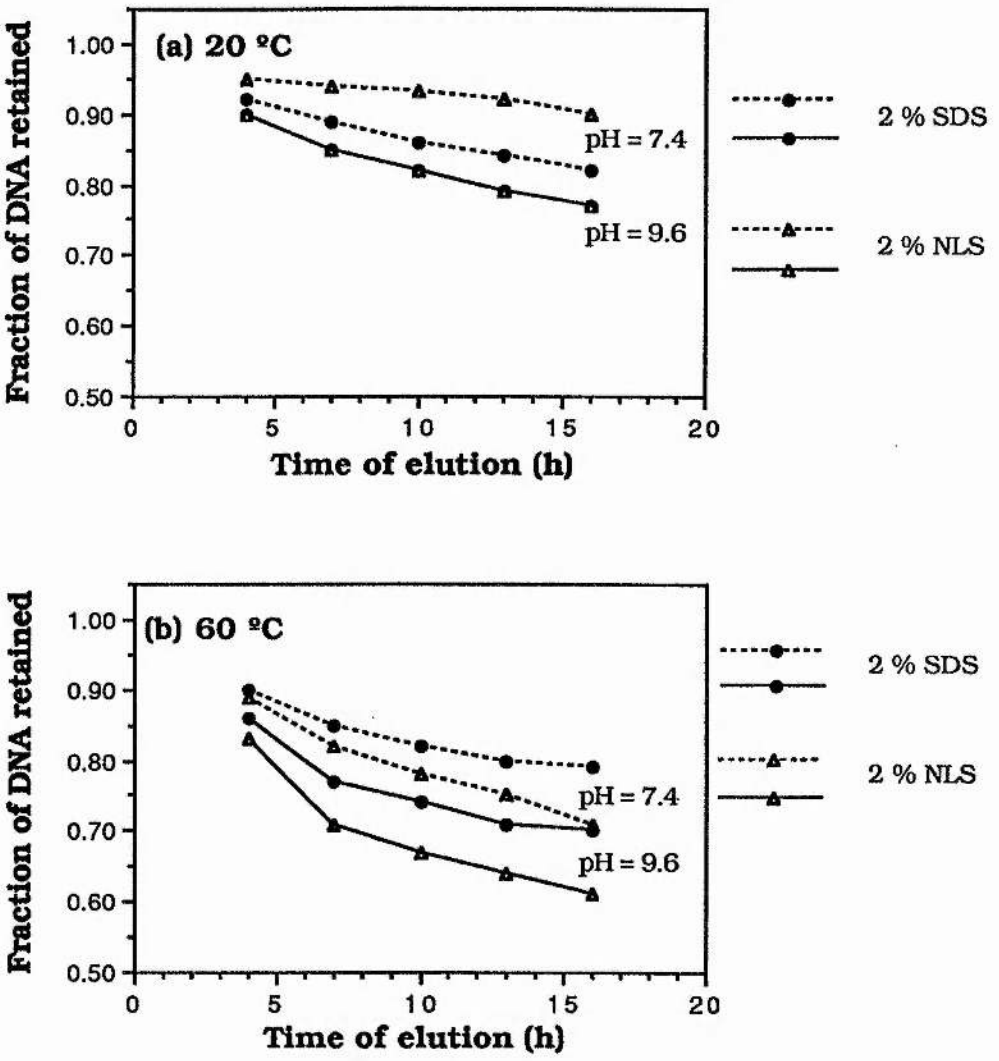


Figure 2.5 The effect of the detergents SDS and NLS, used in the lysis solution, on the elution profiles at pH 7.4 and 9.6 of the DNA of CHO cells exposed to 20 Gy X-rays and lysed for 1 h at either (a) room temperature (20 °C) or (b) at 60 °C.

At the lysis temperature of 20 °C (Fig. 2.5a) more rapid elution was obtained using SDS at pH 7.4, whereas the two detergents gave identical

elution profiles at pH 9.6 (the two graphs are superimposed). By comparing panels (a) and (b) of Fig. 2.5 it can be seen that in all cases (*i.e.* using either detergent) more rapid elution was achieved after lysis at the elevated temperature of 60 °C for 1 h. The background elution values were found to be similar at both temperatures of lysis treatment. This increased elution at 60 °C could be due to heat labile sites in the damaged DNA that would lead to the formation of additional dsb at the elevated temperature (1st L.H. Gray Workshop). From Fig. 2.5(b) it can be seen that increased elution was obtained with NLS rather than SDS at both pH values after lysis treatment at 60 °C.

2.4 Conclusions

The purpose of testing the various lysis and eluting protocols within the range of conditions generally in use, was an attempt to maximize the sensitivity of the non-denaturing filter elution assay. Conditions were sought that would enhance the extent of elution of irradiated DNA, but that would not significantly alter the shape of the elution profiles. This is based on the assumption that should the altered lysis or elution conditions change the physico-chemical properties of the DNA, it would be manifested as a change in the elution kinetics.

After consideration of the results obtained in the experiments described in this chapter, it was decided to standardise on the following experimental protocol: CHO cells, seeded at 1.10^6 cells per flask, were labelled with 3.7 kBq/ml tritiated thymidine and an equimolar amount of unlabelled TdR (1 μmol/l) for 48 h. Cells were irradiated in suspension in MEM at a concentration of $2\text{-}3.10^5$/ml. 5.10^5 cells, diluted in ice-cold PBS were deposited on each polycarbonate filter (2 μm pore size). The

lysis conditions entailed incubation of the cells for 1 h at 60 °C in 1 ml of lysis solution containing; 0.025 mol/l Na_2EDTA, 0.1 mol/l glycine and 0.07 mol/l NLS (adjusted to either pH 7.4 or 9.6) and freshly dissolved proteinase K at 0.5 mg/ml. The DNA was eluted with 40 ml of elution buffer consisting of 0.02 mol/l EDTA (free acid form) and ~0.05 mol/l TPAH and adjusted to pH 7.4 or 9.6 with TPAH. A flow rate of 3 ml/h was maintained and 3 hourly fractions collected over a 15-17 h elution period.

CHAPTER 3

DOSE-RESPONSE AND REPAIR CURVES

3.1 Introduction

3.1.1 Data representation

3.1.2 Dose-response curves

3.1.3 Repair curves

3.2 Materials and methods

3.2.1 Dose-response experiments

3.2.2 Repair experiments

3.3 Results

3.3.1 Dose-response curves

3.3.2 Repair kinetic studies

3.4 Discussion

3.4.1 Non-linear dose-response curves

3.4.2 Biphasic repair kinetics

3.1 Introduction

3.2.1 *Data representation*

A further unresolved aspect of the neutral elution technique has been the manner of data representation.

The initial approach was to present the complete series of elution profiles obtained (Bradley and Kohn 1979), but simpler representations were sought in which a property of the elution profile, rather than the whole profile is plotted against a chosen variable (*e.g.* dose or repair time). The neutral elution profiles are not linear (on a plot of log [fraction of DNA retained] versus elution time) and therefore it was not immediately evident which property of the elution profile would be the best measure of the assay. It is obvious from the literature that great variation exists in the criteria by which the information is extracted from elution profiles. In the earliest reports on the non-denaturing filter elution assay, the authors chose simply to plot the fraction of DNA retained on the filter after a specified elution time as a function of X-ray dose or incubation time (Bradley and Kohn 1979; Woods 1981; Ross and Bradley 1981).

An internal standard, *i.e.* cells irradiated to a fixed dose and added to each sample, is often employed in the neutral elution assay to compensate for variations that might occur between sampling lines. Internal standards usually consist of ^{3}H-labelled cells which can be distinguished from the test cells labelled with ^{14}C. Bradley and Kohn (1979) employed an internal standard in their initial experiments to determine the reproducibility of the assay, but they did not incorporate these values in the calculation of their elution results. Many investigators have utilised an internal standard (of ^{3}H-labelled cells) to compensate for sampling variations by using the elution rate of the ^{3}H-DNA to define a corrected

time scale for the elution of the test DNA (Zwelling *et al.* 1981; Van der Schans *et al.* 1983; Goldenberg and Froese 1985; Wlodek and Hittelman 1987; Fox and McNally 1988; Kelland *et al.* 1988). A similar procedure had routinely been used to present alkaline elution data and is described in detail in Kohn *et al.* (1980).

Radford (1983) took this procedure one step further and used ^{3}H-labelled cells as an internal reference *i.e.* his data were expressed as relative elution values obtained from the ratio:

$$RE = \frac{\text{total } ^{14}C \text{ c.p.m. eluted after 17 h}}{\text{total } ^{3}H \text{ c.p.m. eluted after 17 h}}$$

He reported that in so doing the reproducibility of the assay was markedly improved.

Other authors (*e.g.* Weibezahn and Coquerelle 1981; Maki *et al.* 1986; Wlodek and Hittelman 1987; Coquerelle *et al.* 1987) have used the term relative elution to describe the logarithmic relationship:

$$RE = - \log (x_I/x_C)$$

where I refers to irradiated and C to unirradiated or control cells. The two above mentioned authors however differed in their assignation of the variable x. For Wlodek and Hittelman (1987) x was the fraction of DNA left on the filter when 25 % of the internal standard had been eluted, whereas for Weibezahn and Coquerelle 1981 and Maki *et al.* (1986) x was the mean value of the percentage DNA retained in the filter (*i.e.* the mean of the percentages of DNA retained of all the fractions). The latter authors had not included the use of an internal standard.

More recently, Prise *et al.* (1989a) introduced an even more complicated concept, that of relative log elution (RLE) in an attempt to obtain linear induction curves for the non-denaturing filter assay.

Another function which is used to characterise relative numbers of DNA strand breaks, as assayed by non-denaturing filter elution, is the strand scission factor (SSF), which can be obtained in a number of ways:

$SSF = - \ln(f_I/f_C)$ as does Swiegert *et al.* (1988) or

$SSF = - \log(f_I/f_C)$ as does Murray *et al.* (1988b) or

$SSF = |(f_I/f_C)|$ as does Sigdestad *et al.* (1987)

where f_I and f_C are fractions of DNA remaining on the filter after x hours or volume eluted for the irradiated (I) and unirradiated (C) samples respectively (once again without the inclusion of an internal standard).

Neither did Iliakis and Okayasu (1988) use an internal standard and they chose simply to plot the fraction of DNA eluted after x hours of elution against dose, where the fraction of DNA eluted is obtained by taking [1- fraction of DNA retained] and subtracting the fraction eluted in the unirradiated sample (*i.e.* the background elution values).

Mayer *et al.* (1986; 1989) obtained linear elution profiles under non-denaturing conditions and therefore could use the mean slope of each profile as the chosen parameter.

All the above-mentioned examples serve to illustrate that no standard approach to data representation has been adopted for the non-denaturing filter elution technique. This is possibly due to the lack of understanding of the physico-chemical mechanism of neutral elution and hence the lack of consensus of how to interpret and best represent the results.

Another aspect of neutral elution which is problematic is the lack of accurate calibration. There is very strong evidence indicating that the extent of elution of the DNA reflects numbers of dsb (Bradley and Kohn 1979), but unlike neutral velocity sedimentation (Blöcher 1982) no absolute numbers of dsb can be extracted from the results. Radford and Hodgson (1985) and Peak *et al.* (1988a) have attempted to calibrate the neutral elution method using ^{125}I- UdR (labelled deoxyuridine), on the basis that each ^{125}I decay produces on average one DNA dsb. After incorporation of the ^{125}I into the DNA, the cells were allowed to accumulate a known number of decays and therefore putative dsb during liquid nitrogen storage. Using an estimated average DNA content of the cell line and a X-ray dose that gave an equivalent [fraction of DNA eluted] value to that observed for the known number of ^{125}I-decays, the breakage efficiency values, *viz.* the number of dsb/genome/Gy, could be calculated. The values obtained in this manner were consistent with the estimates obtained from neutral velocity sedimentation (Lehmann and Stevens 1977; Blöcher 1982; Ahnström and Bryant 1982).

In general the calibration of the neutral elution technique has not been widely implemented, probably because ^{125}I was found to give a linear dsb induction curve as compared with a curvilinear X-ray induction curve. This difference in the shape of the induction curves forms part of the on-going argument as to the actual nature of the lesions/dsb that are detected by this assay. The final outcome is that the non-denaturing filter elution technique is mostly used as a comparative assay of relative levels of dsb.

3.1.2 *Dose-response curves*

Many authors obtained a linear dose-response curve for X-ray doses in the range 10 - 200 Gy (Ross and Bradley 1981; Weibezahn and

Coquerelle 1981; Woods 1981; Maki *et al.* 1986; Mayer *et al.* 1986; Sigdestad *et al.* 1987; Murray *et al.* 1988b). Those investigators however who attempted measurements below 10 Gy, found a shoulder in this low dose region (*e.g.* Ross and Bradley 1981; Woods 1981; Sigdestad *et al.* 1987; Wlodek and Hittelman 1987; Swiegert *et al.* 1988) and therefore represented the induction data as a curvilinear plot (*e.g.* Radford 1985; Prise *et al.* 1987; Swiegert *et al.* 1988).

The reason for this shoulder is not yet fully understood (see review by Radford 1988), although the size of the shoulder would seem to be affected by the presence of sulphydryl compounds (Radford 1987b) and position in the cell cycle of the treated cells (Okayasu *et al.* 1988). Based on the results of their respective experiments, Radford (1987b) proposed that the curvilinear dose-response reflected a underlying mechanism of induction of cellular lesions, while Okayasu *et al.* (1988) maintained that the shoulder was due to incomplete denaturation of the chromatin structure of DNA. As discussed previously in Chapter 1 (section 1.6.2) Radford postulated that the shouldered dose-response curve mirrored the shouldered cell survival curve, which implied that cell killing is mainly determined by the induction of dsb. This line of reasoning has been supported by some workers (Prise *et al.* 1987; Kelland *et al.* 1988) and disputed by others (Wlodek and Hittelman 1987; Iliakis and Okayasu 1988; Swiegert *et al.* 1988) depending on the cell line and elution conditions employed. Recently Blazek *et al.* (1989) introduced a new approach by suggesting that the curvilinear dsb dose-response data (measured by neutral elution) could be explained by assuming that the induction of dsb is proportional to the square of the dose.

It is obvious that the dispute as to whether the non-linear induction curve of the non-denaturing filter elution technique is an artefact of the

technique or whether it reveals an underlying mechanism of DNA damage induction, has yet to be resolved.

3.1.3 *Repair curves*

Bradley and Kohn (1979) showed that non-denaturing filter elution could also be used to measure dsb repair. They followed the level of breaks for up to 120 min post-irradiation and their data indicated biphasic repair kinetics with 50 % of the breaks disappearing within approximately 40 min. This result was surprising since neutral velocity sedimentation studies had found a single repair component with a half-time of >1 h (Lehmann and Stevens 1977, Bryant and Blöcher 1980; Blöcher and Pohlit 1982). Numerous groups have subsequently confirmed the measurement of biphasic kinetics using the neutral elution assay *e.g.* Weibezahn and Coquerelle (1981); Woods (1981); Van der Schans *et al.* (1983); Kemp *et al.* (1984); Maki *et al.* (1986); Mayer *et al.* (1986) and Murray *et al.* (1988b). The results of Sigdestad *et al.* (1987) showing biphasic repair measured at pH 9.6 as compared with a single component of repair at pH 7.4 increased the debate, but this finding has subsequently been disputed by other workers who found biphasic kinetics at the lower pH as well (Koval and Kazmar 1988b; Rowley and Kort 1988).

The observation of biphasic repair kinetics has become characteristic of the neutral elution assay, and the disagreement with the apparently first-order kinetics measured with velocity sedimentation has contributed to the controversy surrounding the technique.

3.2 Materials and methods

The cell culture, irradiation and filter elution procedures used, are described in Chapter 2 (sections 2.2.1 and 2.2.2).

3.2.1 *Dose-response experiments*

Initial dose-response experiments included the use of ^{3}H-labelled cells as an internal reference. These cells were seeded and labelled with 3.7 kBq/ml ^{3}H-TdR and 1 µmol/l TdR, separately from the test cells, and after trypsinization they were irradiated to a standard dose, usually 20 Gy. The test cells were labelled with 3.7 kBq/ml ^{14}C-TdR, and irradiated to various doses of up to 80 Gy. Both batches of cells were kept separately, on ice, throughout and only combined just before the cells were aliquoted into ice-cold PBS in the reservoirs of the elution apparatus. The cells were mixed in the ratio of 1.10^{5} ^{3}H-labelled cells to 4.10^{5} ^{14}C-labelled cells. The collected fractions were then analysed for levels of both ^{3}H and ^{14}C-activity on the dual-counting mode of a LKB 1214 Rackbeta scintillation counter.

The dose-response data was then plotted as relative elution (RE) values against dose, where

$$RE = \frac{\text{fraction of } ^{14}\text{C-activity eluted after 16 h}}{\text{fraction of } ^{3}\text{H-activity eluted after 16 h}}$$

(as per Radford 1983) or simply as the fraction of ^{14}C-DNA eluted after 16 h with the background elution value subtracted (for the unirradiated sample), against dose (as per Okayasu and Iliakis 1989).

For reasons that will be explained in section 3.3.1, a single label of ^{3}H-TdR was used in the majority of experiments. The parameter chosen

to quantify the amount of DNA damage was the fraction of ^{3}H-DNA activity eluted from the filter in 16 h. This parameter was chosen under the premise that when the data obtained, using the modified lysis conditions of Okayasu and Iliakis (1989), was plotted in this fashion against radiation dose it would produce a linear dose-response relationship.

3.2.2 *Repair experiments*

Essentially the same procedure as described in Chapter 2 (sections 2.2.1 and 2.2.2) was followed. The only difference was that in the repair experiments the cell suspensions (3 ml in bijou bottles) were kept in a water-bath at 37 °C before irradiation, irradiated to 30 Gy at 37 °C and then returned to the water-bath for the appropriate repair times (5-180 min). The irradiations were staggered such that all samples could be collected at a common time point. To halt repair, samples were poured into a large volume (10 ml) of ice-cold PBS and held on ice until such time as they could be processed further. The only exception was the sample of unrepaired cells, which was transferred to ice approximately 0.5 h before irradiation, irradiated on ice and immediately afterwards poured into ice-cold PBS and held on ice until it could be processed further.

The above procedure had to be altered slightly for the long-term repair experiments where cells were incubated at 37 °C for up to 8 h post-irradiation. The unrepaired and the t = 0.5 h samples (*i.e.* cells that were allowed to repair for $^{1}/_{2}$ h) were processed as described above, whereas the other samples were processed as follows; the cell suspensions (3 ml in bijou bottles) were held at 37 °C in the water-bath before irradiation and irradiated to 50 Gy at 37 °C. The samples were then transferred to tissue-culture petri dishes and after addition of a further 2 ml MEM, the samples were placed in a 37 °C, 5 % CO_2

humidified incubator for the various repair times. Once again, the irradiations were staggered such that all samples could be collected at a common time point, at which time the cells were trypsinized using cold trypsin/EDTA, resuspended in 2-4 ml cold MEM and shortly afterwards introduced into the ice-cold PBS (in the elution reservoirs).

The neutral elution procedure (as described in section 2.2.2) was followed under the conditions stipulated in section 2.4 and performed at both pH 9.6 and 7.4.

The data of the repair experiments have been plotted as log [fraction of DNA eluted at 16 h] versus dose. The same data was also plotted as % breaks (assumed to be dsb) rejoined with time. These values were obtained using the formula:

$$\% \text{ breaks rejoined} = (1 - x_t/x_o).100$$

where x_t = the fraction of DNA eluted after repair time t and

x_o = the fraction of DNA eluted in the unrepaired sample

(background elution values had been subtracted in both cases).

3.3 Results

3.3.1 *Dose-response curves*

A series of the elution profiles of DNA obtained after CHO cells had been exposed to increasing doses of X-irradiation, as measured by neutral elution at pH 9.6 and under the lysis and eluting conditions stipulated in section 2.4, is shown in Fig. 3.1. For this experiment the test cells were pre-labelled with ^{14}C-TdR and an internal standard of ^{3}H-labelled cells, irradiated to 20 Gy, was added to each sample.

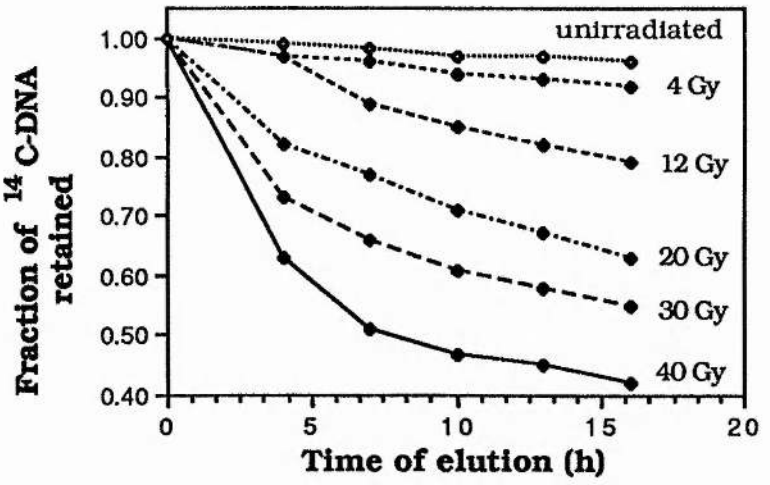
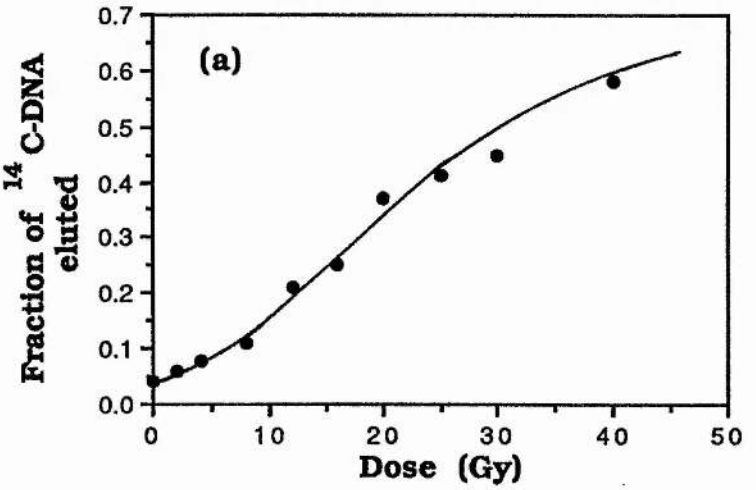


Figure 3.1 The elution profiles obtained with non-denaturing filter elution at pH 9.6 of the DNA of CHO cells exposed to increasing doses of X-irradiation.

It can be seen that the rate of elution of the DNA from the filter increased with dose and hence the fraction of ^{14}C-DNA retained after 16 h of elution diminished with increasing dose. In Fig 3.2 (a) the fraction of ^{14}C-DNA eluted in 16 h, taken from the elution profiles in Fig. 3.1, is plotted against X-ray dose and in Fig. 3.2 (b) the relative elution values (as per Radford 1983) are plotted against dose. Both curves have been fitted by eye.



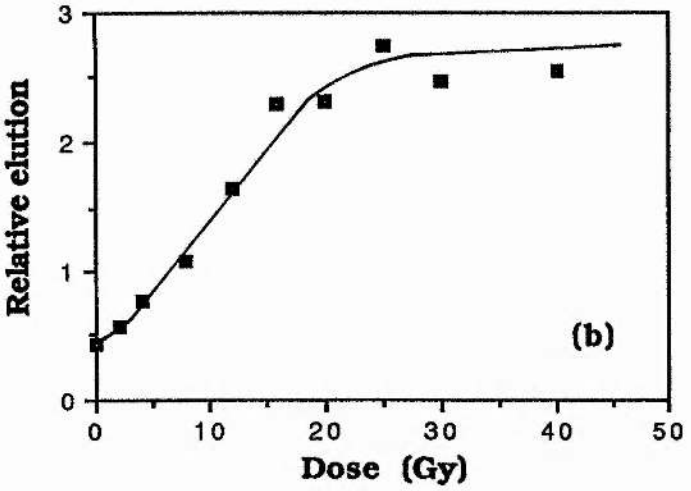


Figure 3.2 Examples of dose-response curves of CHO cells as measured by neutral elution at pH 9.6. In (a) the fraction of ^{14}C-DNA eluted after 16 h is plotted against dose and in (b) the relative elution value (see section 3.2.1) is plotted against dose.

Clearly both the fraction of [^{14}C-DNA eluted in 16 h] and the relative elution values exhibited an increase with increasing X-ray dose. Both figures show a sigmoidal dose-response *i.e.* a shoulder in the low dose region (0-5 Gy) followed by a linear portion and then a tendency of the curve to plateau out at the higher doses. This tendency is far more pronounced in Fig. 3.2 (b) where the curve flattens out at a dose of approximately 20 Gy. I suspected that this was due to the elution of the ^{3}H-labelled standard cells which seemed to increase with increasing X-ray dose given to the ^{14}C-labelled test cells. To substantiate this, the fraction of ^{3}H-DNA eluted, where the ^{3}H-labelled cells were exposed to a X-ray dose of 20 Gy throughout, was plotted against the dose received by the ^{14}C-labelled cells (Fig. 3.3).

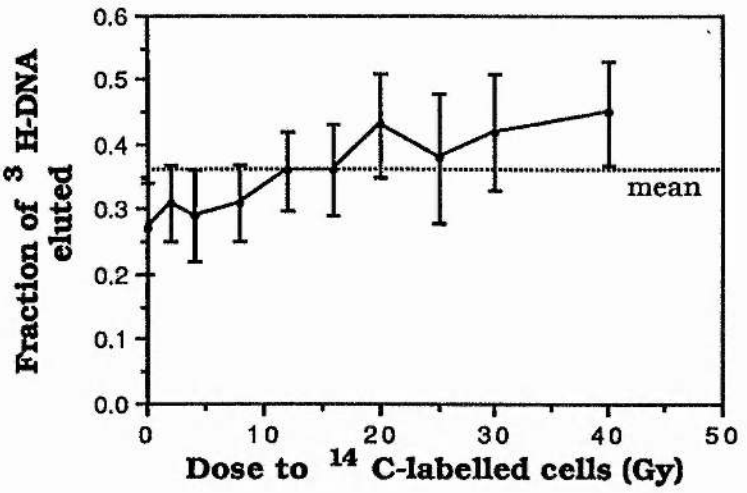


Figure 3.3 Graph showing the fraction of ^{3}H-DNA eluted (from the internal standard of cells exposed to 20 Gy) plotted against the X-ray dose given to the ^{14}C-labelled cells. The data points represent the mean of 4 experiments and the vertical bars the standard error of mean values.

Clearly the increase in the rate of elution of the ^{14}C-DNA of the test cells with dose caused an increase in the elution of the ^{3}H-DNA. This finding undermined the purpose of the internal standard, the use of which was therefore abandoned. A single radioactive label, usually ^{3}H-TdR, was used in all subsequent experiments at the risk of not observing variations between sampling lines. To avoid reoccurring erroneous elution results the samples were alternated with respect to the 10 sampling lines.

The dose-response curve for CHO cells obtained by neutral elution performed at pH 9.6, after 8 repeat experiments, is shown in Fig. 3.4. The fraction of DNA eluted was obtained from the fraction of ^{3}H-DNA eluted minus the background elution value. The curve has been fitted by eye.

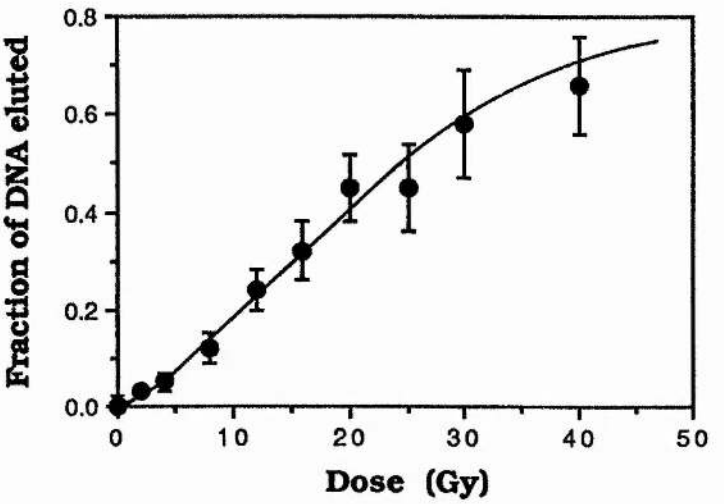


Figure 3.4 Dose-response for CHO cells as measured by neutral elution at pH 9.6. The data points represent the mean of 8 experiments and the vertical bars the standard error of mean values.

A concave 'shoulder' in the low dose region (0-5 Gy) is evident, this is followed by a linear increase in fraction of DNA eluted with dose up to about 40 Gy, after which the curve reaches a plateau. The single data point at 40 Gy does not unequivocally prove the existence of the plateau, but the plateau was based on the results of preliminary dose-response experiments in which doses of up to 80 Gy were used (the additional data points are shown in Fig. 3.5). It was decided to limit the detailed investigation to the shoulder and linear portions of the dose-response curve and hence the sparsity of data at doses greater than 40 Gy.

The dose-response for CHO cells as measured by neutral elution performed at pH 7.4 is shown in Fig. 3.5. The data points of the dose-response assay at pH 9.6 (data taken from Fig. 3.4) have been included and are denoted by the dashed line to serve as a comparison. The data points above 50 Gy, for both pH values, are the results of a single experiment and were included to illustrate the plateau. Once again the curves have been fitted by eye.

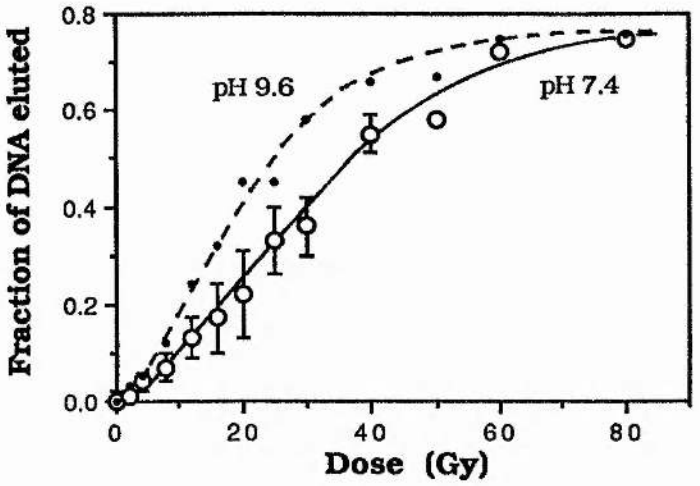


Figure 3.5 Dose-response for CHO cells as measured by neutral elution at pH 7.4. The data points represent the mean of 3 experiments and the vertical bars the standard error of mean values. The dashed line shows the data taken from Fig. 3.4.

Clearly the extent of elution of the DNA at each X-ray dose is less at pH 7.4 than at 9.6, but the shape of the induction curve remains essentially the same. The shoulder in the low dose region is also evident at pH 7.4, although it is less pronounced than at pH 9.6. The dose at which the curves flattened out in the assays at the two pH values are different but note that the plateau occurs at similar elution levels, *viz.* at a fraction of DNA eluted of ~0.75. This signifies that 15-20 % of the DNA, after taking the background elution value of 5-10 % into account, remains on the filter even after high doses of radiation.

3.3.2 *Repair kinetic studies*

Repair kinetics after a X-ray dose of 30 Gy as measured by neutral elution at pH 9.6, is shown in Fig. 3.6.

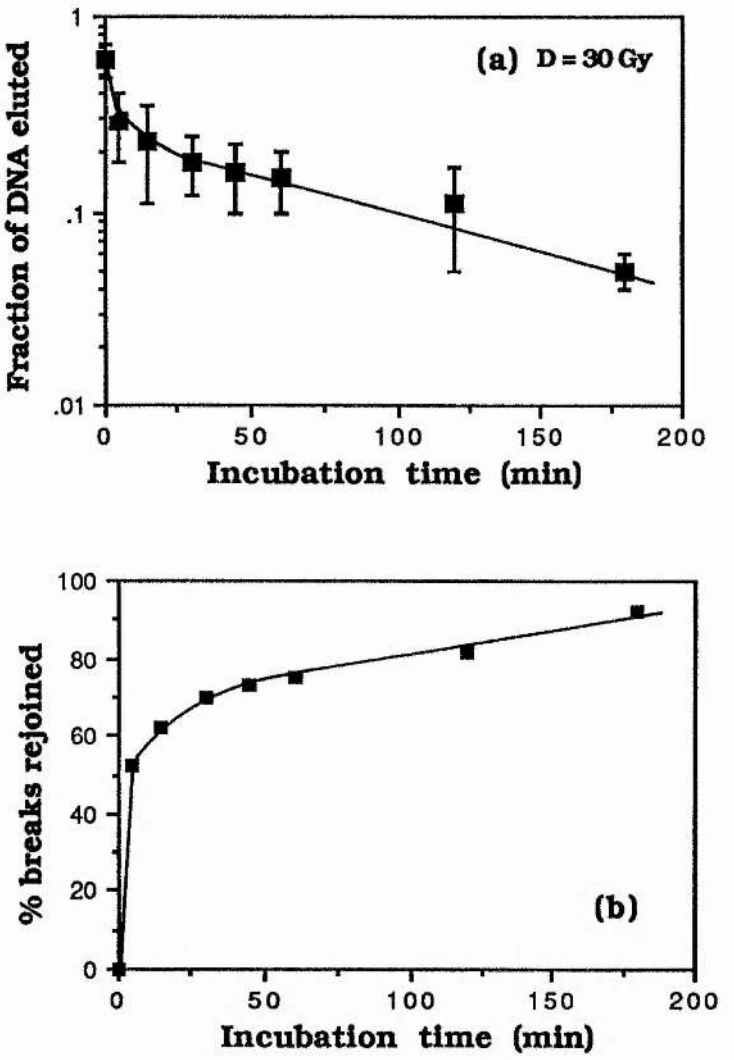


Figure 3.6 The kinetics of disappearance of DNA strand breaks in CHO cells as measured by the neutral elution technique (pH 9.6) as a function of incubation time after X-ray exposure (30 Gy). The data points represent the mean of 10 experiments and the vertical bars the standard error of mean values. In (b) the data from (a) is plotted as % breaks rejoined (see section 3.2.2).

The decrease in the extent of elution of the DNA at increasing incubation times indicates that rejoining of the breaks had occurred. The curves are typically biphasic (as evidenced by the two component logarithmic graph) and rough estimates of the repair half-times ($t_{1/2}$) of the fast and slow components are 6 and 120 min respectively. In panel (b) the data from (a) is plotted as % breaks rejoined (see section 3.2.2). These results

show that approximately 90 % of the breaks had rejoined after a repair time of 3 h.

The rate of repair of dsb in CHO cells after an X-ray dose of 30 Gy as measured by neutral elution at pH 7.4 is shown of Fig 3.7.

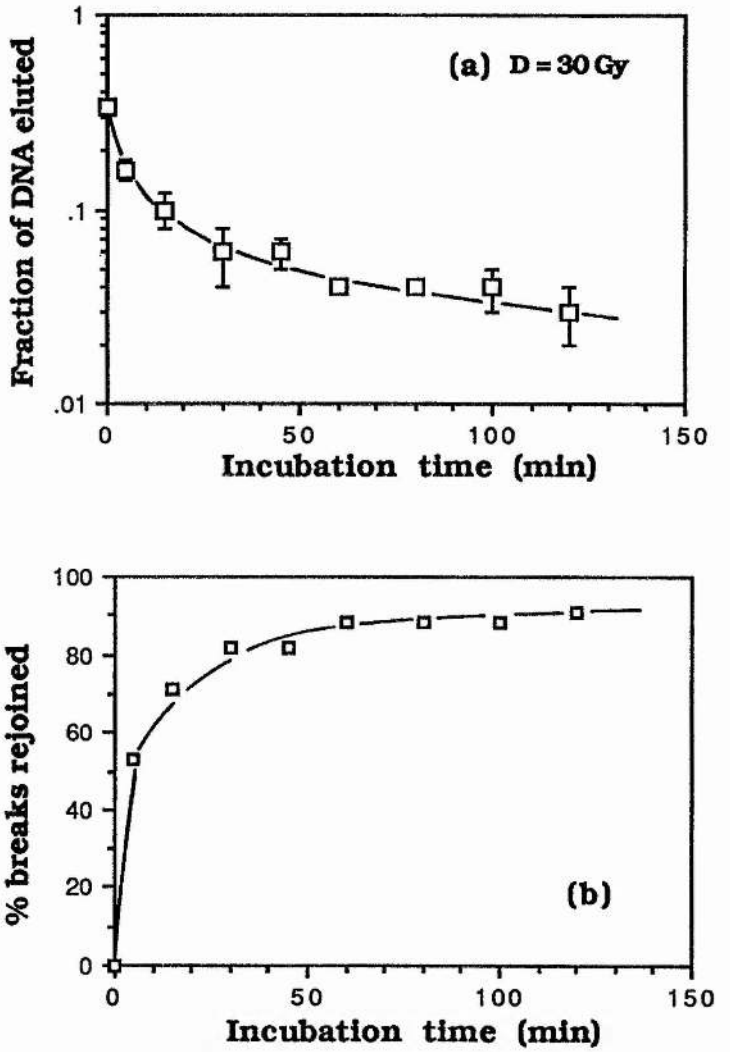


Figure 3.7 The kinetics of disappearance of DNA strand breaks in CHO cells as measured by the neutral elution technique (pH 7.4) as a function of incubation time after X-ray exposure (30 Gy). The data points represent the mean of 2 experiments and the vertical bars the standard error of mean values. In (b) the data from (a) is plotted as % breaks rejoined (see section 3.2.2).

Typical biphasic repair kinetics were also obtained at this pH (7.4), and rough estimates of the repair $t_{1/2}$ are 5 and 100 min for the fast and slow components respectively. In panel (b) the data from (a) are plotted

as % breaks (assumed to be dsb) rejoined with time. From this graph it can be seen that approx 90 % of the breaks had rejoined after a repair time of 2 h.

Fig. 3.8 shows the repair kinetics of CHO cells after a dose of 50 Gy as measured by neutral elution at pH 9.6 over a longer time course namely 0.5 - 8 h.

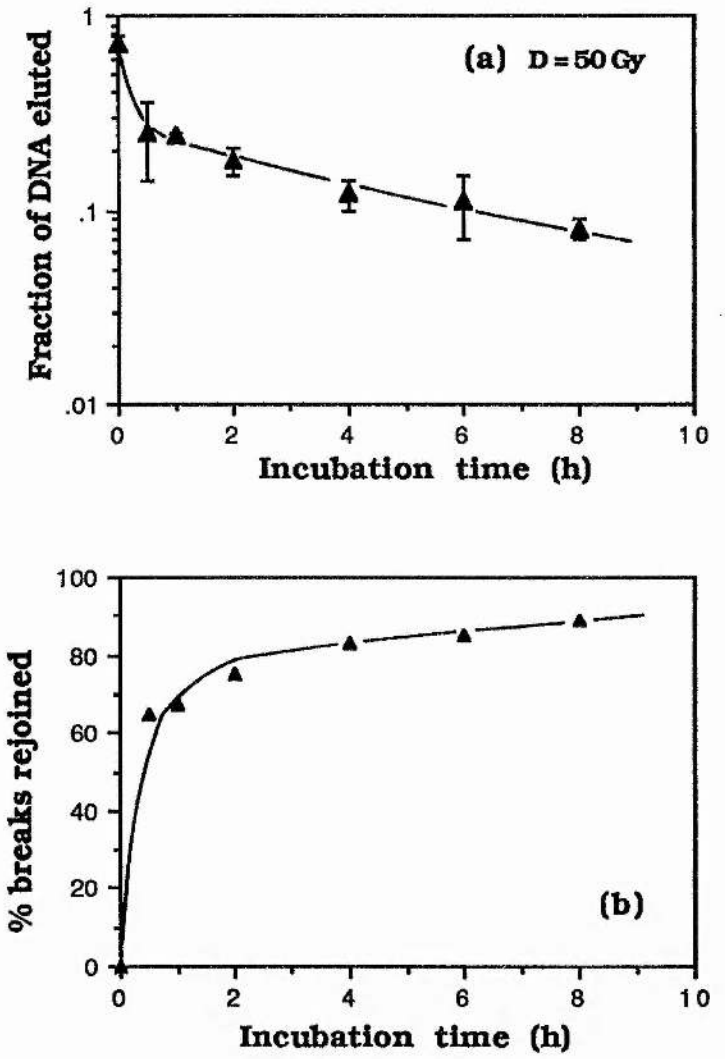


Figure 3.8 The kinetics of disappearance of DNA strand breaks in CHO cells as measured by the neutral elution technique (pH 9.6) as a function of incubation time after X-ray exposure (50 Gy). The data points represent the mean of 2 experiments and the vertical bars the standard error of mean values. In (b) the data from (a) is plotted as % breaks rejoined (see section 3.2.2).

It can be seen from Fig. 3.8 that the number of breaks continue to decrease over the 8 hour incubation time after X-ray exposure. This curve represents more than one repair component, but the data points are insufficient to ascertain whether there are 2 or possibly 3 repair components. There does however seem to be a very slow component of repair with a $t_{1/2}$ in the range of 4-6 h. In panel (b) the data from (a) is plotted as % breaks rejoined with time and this graph shows that approximately 90 % of the breaks (assumed to be dsb) had rejoined after 8 h post-exposure incubation.

3.4 Discussion

3.4.1 *Non-linear dose-response curves*

^{3}H-labelled cells were included in initial experiments as an internal standard which would serve to compensate for any variations between the sampling lines. According to Bradley and Kohn (1979) and Evans *et al.* (1987) the extent of ^{14}C-DNA elution did not affect the elution of the ^{3}H-DNA if the cell number per filter was kept below 1.10^{6}. My results (Fig. 3.3) however show that under the altered lysis and eluting conditions (described in section 2.4) and at a cell number of 5.10^{5} per filter, the elution of the ^{3}H-DNA was affected by the increasing rate of elution of the ^{14}C-DNA with X-ray dose. This undermined the purpose of the ^{3}H-standard and its use was therefore abandoned. By doing this the question of the manner of data representation was simplified and thus all data are simply presented as the fraction of DNA-associated radioactivity eluted from the filter in 16 h with the background elution value subtracted.

When the dose-response curve shown in Fig. 3.4 is compared to the dose-response curves obtained by other workers using neutral elution at

pH 9.6 (*e.g.* Bradley and Kohn 1979; Woods 1981; Sigdestad *et al.* 1987; Murray *et al.* 1988b), the modified lysis conditions employed here clearly resulted in greater sensitivity of the assay.

Figure 3.4 shows that when the fraction of DNA eluted after 16 h at pH 9.6 is plotted against dose, a sigmoidal dose-response curve was obtained. A shoulder in the low dose region (0-5 Gy) is followed by a linear increase in the fraction of DNA eluted up to 40 Gy after which the curve plateaus out. The shape of the dose-response curve obtained at pH 7.4 was also sigmoidal (Fig. 3.5); showing a shoulder in the 0-5 Gy region, followed by a linear increase in the fraction eluted extending up to 60 Gy and for doses greater than 60 Gy a plateau was observed. Sigdestad *et al.* (1987) and Murray *et al.* (1988b) reported that they obtained a curvilinear dose-response relationship at pH 9.6 but that at pH 7.4 they obtained linear curves. Closer inspection of their results obtained at pH 7.4 however shows a sparsity of data at low doses and relative insensitivity of the assay under the conditions they employed. On the other hand Radford (1988), Okayasu and Iliakis (1989) and Prise *et al.* (1989c) found that the dose-response at the lower pH also exhibited a shoulder and thus concluded that the shoulder and hence the shape of the dose-response curve was not an artefact of the elution pH.

By employing more rigorous lysis conditions (*viz.* using NLS rather than SDS and the elevated lysis temperature of 60 °C) Okayasu and Iliakis (1989) found that they could eliminate the shoulder (which they thus attributed to incomplete denaturation of the higher order chromatin structure). The dose-response curves presented here were obtained using the modified lysis conditions of Okayasu and Iliakis (1989) and yet a small shoulder was nevertheless still observed.

Other than the shoulder in the low dose region, a tendency for the dose-response curves to plateau out at large doses was also observed (see

Fig. 3.5). This occurred at doses greater than 40 Gy for the assay at pH 9.6 and > 60 Gy at pH 7.4. This tendency was found to be more pronounced when the elution values of the ^{3}H-labelled reference cells were included in the calculation of the relative elution values, as seen in Fig. 3.2. Other authors who had also reported a strong tendency for the induction curve to flatten out at higher doses, had in fact made use of an internal reference and this might explain their results (Prise *et al.* 1989a; Kelland *et el.* 1988). On the other hand, since an internal standard was not employed here, the presence of the plateau would rather seem to suggest that a certain proportion of the DNA would not elute from the filter. It is interesting to note that this 'unelutable' proportion of 15-20 % of the total amount of DNA is independent of pH employed. This plateau could be the result of a portion of DNA that is bound to the polycarbonate filter or a portion of DNA that has retained its higher order structure in a DNA-protein complex and is therefore unable to pass through the pores in the filter.

The fraction of DNA eluted after 16 h at pH 7.4 is less than that measured at pH 9.6 for the same X-ray dose (Fig. 3.6), indicating that the technique is somewhat less sensitive at the lower pH. The pH effect observed here is however not nearly as marked as found by other workers (*e.g.* Evans *et al.* 1987; Murray *et al.* 1988a). Using the more rigorous lysis conditions of 2 % NLS and a temperature of 60 °C, Okayasu and Iliakis (1989) reported that they had succeeded in eliminating the pH effect. Even though the modified lysis conditions of Okayasu and Iliakis (1989) were employed here, the pH effect was still observed. One obvious difference in the protocols used was the composition of the eluting buffer: Okayasu and Iliakis (1989) had followed the protocol of Bradley and Kohn (1979) and used a tris solution to elute the DNA, whereas a TPAH elution buffer was used here. Evans *et al.* (1987) and Murray *et al.* (1988a), who

reported finding a marked pH effect, had in fact also used a TPAH eluting buffer for the neutral elution assay. This is however a tentative correlation and it is not clear how the composition of the eluting solution could explain the pH effect.

Two contradicting theories have been postulated to explain the difference in sensitivity of the assay at the two pH values. Radford (1988) attributed the lower elution at pH 7.4 to incomplete removal of DNA-bound proteins, whereas Evans *et al.* (1986) maintained that the greater elution at the elevated pH of 9.6 was due to additional alkali-labile lesions that would not occur at the normal intracellular pH. There seems to be strong evidence in favour of both these theories. On the one hand, Flick *et al.* (1989) have shown that the increased elution at pH 9.6 is observed for X-ray induced damage only and notably absent in restriction enzyme induced damage, which is strong evidence in favour of the alkali-labile hypothesis of Evans *et al.* (1987). On the other hand, Okayasu and Iliakis (1989) have shown that by altering the lysis conditions they could eliminate the pH effect on the elution of X-irradiated DNA, which is strong evidence in support of Radford's theory.

3.4.2 *Biphasic repair kinetics*

The repair curves at both pH 9.6 (Fig. 3.6) and pH 7.4 (Fig.3.7) show characteristic biphasic repair kinetics, exhibiting a fast component with half-time of 5-7 min and a slower component with $t_{1/2}$ of 100-120 min. Biphasic repair kinetics for the non-denaturing filter elution assay have been obtained by several authors at pH 9.6 (Woods 1981; Weibezahn and Coquerelle 1981; Sigdestad *et al.* 1987) and at pH 7.4 (Schwartz *et al.* 1987; Koval and Kazmar 1988b; Fox and McNally 1988). The above estimates of the half-times of repair are in reasonable agreement with those obtained for mammalian cells by other authors using the neutral

elution technique at pH 9.6, namely that of 2-10 min for the rapid component and $t_{1/2}$ of 30-120 min for the slow component as measured up to 3 h post-exposure (Weibezahn and Coquerelle 1981; Radford 1983). At this point it is important to mention that the time taken to irradiate the cells to 30 Gy (~5 min) or 50 Gy (~9 min), at 37 °C, is not insignificant when the numbers of dsb remaining at repair times of less than 60 min post-exposure are considered. As a consequence, the initial fast repair could possibly be somewhat slower than would appear to be the case from the $t_{1/2}$ estimates of the fast component given here.

At both pH values approximately 50 % of the breaks had rejoined within 5 min of irradiation, which would indicate that the measurement of the fast repair kinetics is independent of the pH. Marginally different rates of rejoining were however measured for the slow component at the two pH values; approximately 90 % of repair had taken place after an incubation time of 60 min in the pH 7.4 assay, whereas this level of rejoining was only attained after 180 min at pH 9.6. Ball-park estimates (more data points are needed for more accurate estimates) of the half-times of repair are given in Table 3.1.

pH of filter elution assay	$t_{1/2}$ for fast repair component	$t_{1/2}$ for slow repair component
9.6	~ 6 min	~ 120 min
7.4	~ 5 min	~ 100 min

Table 3.1 Ball-park estimates of the repair half-times for CHO cells after exposure to 30 Gy obtained from Figs. 3.6 and 3.7.

In contrast to the data presented here, Schwartz *et al.* (1987) and Koval and Kazmar (1988b) reported no difference in the rates of repair as assayed at the two pH values of 9.6 and 7.4. Although good reproducibility

was obtained in the repair experiments performed at pH 7.4, further repeat experiments are necessary to ascertain the significance of the difference in repair kinetics at the two pH values.

Koval and Kazmar (1988b) reported that the rate of repair measurement (in V79 cells) at both pH 9.6 and 7.4, depended on the choice of eluting solution. They detected slower repair kinetics with the TPAH buffer than with the tris solution. They thus proposed an interaction between the lesions and the eluting solution, for example the existence of a repair intermediate which is destabilised only in the TPAH solution. The rate of repair measured here using the TPAH buffer (Figs. 3.6 and 3.7) was faster than that measured by Koval and Kazmar (1988b) using the same eluting buffer (but different lysis conditions), and show kinetics similar to the tris solution results of the above authors (although caution should be adopted when comparing the repair kinetics of different cell types as their repair characteristics might be disparate). Repair experiments would need to be performed using the tris eluting solution to definitively show no difference under the modified lysis conditions employed here.

The half-time of the slow component of repair is not dissimilar to the half-time of dsb repair of 2-4 h measured in Ehrlich ascites tumour (EAT) cells by velocity sedimentation (Bryant and Blöcher 1980), while the $t_{1/2}$ of the fast component is not unlike that of ssb repair in EAT or CHO cells (Bryant *et al.* 1984; Costa and Bryant 1988). Ahnström (Comment on Radford 1985) and Hutchinson (1989) have, on the basis of the similar repair rates, proposed that the fast repair component was due to ssb rejoining (assuming that the presence of ssb could affect the elution properties of DNA under non-denaturing conditions). Weibezahn and Coquerelle (1981) and Woods (1981) had put forward an alternative

explanation of the biphasic repair kinetics of the neutral elution assay. They proposed that the rapid component represented a process of ligation and suggested that the slow component reflected a type of repair that required a more complex process, possibly recombination. This interpretation suggests that the biphasic kinetic reflects two different mechanisms of repair which could in turn suggest that the neutral elution assay detects at least two types of lesions or dsb.

When comparing Figs. 3.6 and 3.8 a discrepancy arises in the results of the long (0.5-8 h) and short (5-180 min) term repair experiments. From Fig. 3.6 one would expect approximately 90 % of the dsb to be rejoined by 3 h post-irradiation, whereas Fig. 3.8 shows only 80 % repair at 3 h. Although it is difficult to judge the significance of this difference, considering the errors on the mean values vary between 1.5 and 8 %, it is possible that the slower rate of repair detected after 8 h was due to the higher dose used (50 Gy vs 30 Gy). Possible dose dependence of the $t_{1/2}$ of the slow component of repair would support the saturable repair model proposed by Goodhead (1985) and Wheeler (1987), but Radford (1987a) and Swiegert *et al.* (1989) have reported finding no dose effect in repair experiments following exposure in the 20 to 50 Gy dose range.

Investigation of the repair kinetics over longer incubation times (Fig. 3.8) interestingly revealed the possible presence of a very slow component of repair with a half-time of between 4 and 6 hours. The repair would need to be followed over even longer incubation times to ascertain whether this represents a third repair component or whether it is just an extension of the known slow component. Rowley and Kort (1988) using the neutral elution assay found a continual decrease in numbers of dsb up to 6 h post-irradiation (after 20 Gy) which is further evidence that dsb repair is not complete within 2-3 h as would appear to be the case from the short term (0-3 h) studies. Assuming that there are

only two repair components, an estimate of the $t_{1/2}$ of the slow component from Fig. 3.8 is in the vicinity of 240 min. Rowley and Kort (1988) had obtained an estimate of $t_{1/2}$=170 min for the slow component of repair (followed over 6 h) which is also significantly longer than estimates obtained by other workers of 0.5-2 h (*e.g.* Radford 1983). It is possible that refinements in the technique have improved sensitivity such as to enable the detection of the small numbers of dsb remaining at the longer incubation times, and hence the 'appearance' of a very slow component. Perhaps it is possible that this very slow component (rather than the repair component with $t_{1/2}$ of 30-120 min) represents the same type of repair that is detected by velocity sedimentation for which a $t_{1/2}$ of repair of between 2-4 h was measured (Bryant and Blöcher 1980).

Chapter 4

EXPERIMENTS USING DNA SYNTHESIS INHIBITORS

4.1 Introduction

4.1.1 The inhibitory action of ara A and ara C

4.1.2 The DNA unwinding assay

4.2 Materials and Methods

4.2.1 EAT cell culture, labelling and irradiation

4.2.2 DNA unwinding assay and hydroxylapatite chromatography

4.2.3 Non-denaturing filter elution (pH 9.6)

4.2.4 DNA synthesis assay

4.3 Results

4.3.1 Inhibition of DNA synthesis

4.3.2 DNA unwinding results

4.3.3 Non-denaturing filter elution (pH 9.6) results

4.4 Discussion

4.1 Introduction

One approach to investigate the nature of the lesions that are detected by non-denaturing filter elution, was to measure the effect of DNA synthesis inhibitors on the repair of these lesions. This study was based on the work of Bryant and Blöcher (1982) and Iliakis and Bryant (1983) who found that β-ara A and β-ara C inhibited dsb repair in Ehrlich ascites tumour (EAT) cells as measured by the neutral velocity sedimentation and DNA unwinding techniques. To determine the effect of the above inhibitors on repair of DNA damage as measured by neutral elution (pH 9.6) and to enable a meaningful comparison between these results and those of DNA unwinding, it was decided to use the same cell line *viz.* EAT cells. In this chapter the DNA unwinding results using X-irradiated EAT cells exposed to either ara A or ara C have been verified and compared with the results of the non-denaturing filter elution technique (pH 9.6).

4.1.1 *The inhibitory action of ara A and ara C*

9-β-D-arabinofuranosyladenine (ara A) and 1-β-D-arabinofuranosylcytosine (ara C) are nucleoside analogues of deoxyadenosine and deoxycytosine respectively and are known to strongly inhibit DNA synthesis. These agents have been used in chemotherapy as antitumour cytostatic agents (Ortiz *et al.* 1972) and as anti-leukaemic drugs (Gale and Foon 1986).

In vivo and *in vitro* these nucleoside analogues are readily taken up into the cell and converted to the corresponding triphosphates *viz.* ara-ATP and ara-CTP (Graham and Whitmore 1970; Müller *et al.* 1975). In *Escherichia coli* cells ara A was found to be incorporated into the cellular DNA by the DNA polymerase β where it acted as a chain terminator thus

inhibiting DNA synthesis (Ohno *et al.* 1989). In mammalian cells however the evidence suggests that the inhibitory action is not due to incorporation and chain termination but rather due to inhibition of the DNA polymerases by the triphosphate compound of the analogues (Furth and Cohen 1967; Graham and Whitmore 1970; Müller *et al.* 1975; Dicioccio and Srivastava 1977). Some workers have shown greater inhibition of polymerase α (Müller *et al.* 1977; Okura and Yoshida 1978; Stammberger *et al.* 1989), whereas others have found both polymerases α and β to be equally inhibited (Dicioccio and Srivastava 1977) by ara A or ara C. Thus it becomes evident that these drugs might have a specificity towards one of the polymerases, which would determine the mechanism of their inhibition.

Generally polymerase α is regarded responsible for semi-conservative DNA synthesis whereas polymerase β was found to be involved in DNA repair replication (Spiro *et al.* 1982; Pedrali-Noy and Spadari 1979; Collins 1987; Stammberger *et al.* 1989). Miller and Chinault (1982) found both polymerases α and β be involved in DNA repair, but with a specificity for the type or 'patch-size' of the DNA damage; where polymerase β is responsible for 'short-patch' repair *i.e.* a gap of 1-5 nucleotides, while polymerase α requires a gap of 25-50 nucleotides. This model has been supported by the results of Wang and Korn (1980) and Cleaver (1984). X-ray damage mainly involves short-patch repair (Painter and Young 1972; Fox and Fox 1973) which implicates polymerase β rather than α (Iliakis *et al.* 1982; Mirzayans *et al.* 1988), although not exclusively (Waters *et al.* 1981).

At a cellular level it is known that ara A and ara C inhibit the proliferation of cultured eukaryotic cells (Müller 1979; Iliakis 1980;1981; Iliakis and Bryant 1983; Iliakis *et al.* 1985). At the chromosomal level the effect of the inhibitors are evident in the enhancement of radiation

induced damage as measured by metaphase chromosomal aberrations (Preston 1980), anaphase bridges and fragments (Bryant 1983), chromatid aberrations (Mozdarani and Bryant 1987;1989) and PCC (Iliakis *et al.* 1988b). The most important finding and the one upon which this work is based is the inhibition of dsb repair in X-irradiated EAT cells by β–ara A (Bryant and Blöcher 1982) and β–ara C (Iliakis and Bryant 1983), as determined by neutral velocity sedimentation and DNA unwinding.

4.1.2 *The DNA unwinding assay*

The principle upon which the detection of dsb by the DNA unwinding technique is based, differs greatly from that of the non-denaturing filter elution assay. Whereas with the latter technique the DNA at pH 9.6 remains native, under the mild alkali conditions (0.03 mol/l NaOH; pH 12) employed in the lysis step of the unwinding technique, the double-stranded nature of the DNA is progressively lost. The overall rate of the unwinding in mammalian DNA was found to be dependant on the number of free end points within the DNA strand *i.e.* the number of DNA strand breaks (Ahnström and Erixson 1973). Thus after a fixed duration of alkali treatment, neutralization and sonication (to prevent renaturation) the relative amount of DNA in single-stranded (ss) form is proportional to the number of strand breaks. Alternatively the relative amount of double-stranded (ds) DNA is indicative of the amount of DNA that has remained undamaged.

Mechanistically speaking the DNA unwinding technique cannot distinguish between ssb and dsb and therefore a total number of breaks is measured *i.e.* ssb plus dsb. Bryant and Blöcher (1982) devised a procedure that would enable the distinction between ssb repair and dsb repair based on the repair kinetic differential *viz.* that ssb repair occurs at

a considerably faster rate than dsb repair: The DNA unwinding measurements at short incubation times (0-1 h) after a small X-ray dose (8 Gy) reflects almost entirely ssb repair, whereas the results obtained at longer incubation times (2-8 h) after a larger X-ray dose (50 Gy) reflects mainly dsb repair. The kinetics of repair of DNA strand breaks measured by the unwinding method after the first 2 h of incubation (*i.e.* assumed to be dsb repair), were found to be identical to those for dsb repair measured by neutral velocity sedimentation, so validating the unwinding method for the monitoring of dsb repair. A disadvantage of this kinetic method is that the repair of dsb between time zero and 2 h cannot be measured.

4.2 Materials and methods

4.2.1 *EAT cell culture, labelling and irradiation*

Ehrlich ascites tumour (EAT) cells are a well characterised mouse tumour cell line that can be grown in suspension. The composition of the medium for suspension cultures is described in Iliakis and Pohlit (1979). Cells were kept in exponential growth by reducing the cell concentration from 8.10^5/ml to 2.10^5/ml daily.

Cells at 2.10^5/ml (in 40 ml) were incubated in vertically standing 75 cm^2 plastic flasks (Sterilin) for 24 h in the presence of ^{3}H-TdR (tritiated thymidine, 1.59 TBq/mmol). 37-74 kBq per flask (in 40 ml) was used for the DNA unwinding experiments and 74-148 kBq for the neutral elution experiments. Equi-molar amounts (1-2 μmol/l) of cold thymidine were added to facilitate the uniform uptake of radioactive thymidine.

Cells were diluted to a concentration of 2-10.10^5/ml in medium and 3 ml aliquots in 5 ml plastic bijou bottles were irradiated at a dose-rate of 5.8 Gy/min. In the case of dose-response experiments, cell samples were held on ice for 0.5-1 h before irradiation, irradiated at 0 °C and returned to ice until they could be processed further. For the DNA unwinding experiments the cells were chased in non-radioactive medium prior to irradiation, which involved centrifuging the cells out of the labelled medium and replacing it with unlabelled medium for 4-5 h. This reduced the observed background level of breaks by minimising the detection of unwinding from replication forks (Costa 1987).

For the short-term (0-3 h) repair experiments the cell samples (3 ml in bijou bottles) were held in a 37 °C water bath for 0.5-1 h before irradiation, irradiated at 37 °C and returned to the water bath for the appropriate repair times. Cells were irradiated to 8 Gy for the short-term DNA unwinding repair experiments and to 30 Gy for the neutral elution repair experiments. The irradiations were staggered such that all samples were collected at a common time point, at which time the cells were poured into ice-cold PBS (neutral elution) or ice-cold saline (DNA unwinding), and were kept on ice until further processing.

For the DNA unwinding long-term (2-8 h) repair experiments, the cells were irradiated to 50 Gy at 37 °C and then plated out at 2.10^5 cells per petri dish (non-tissue culture) in 5 ml of medium. The samples were irradiated consecutively, plated out and placed in a humidified, 5 % CO_2 incubator at 37 °C. After the appropriate incubation time, samples were removed and once the cells had been loosened by pipetting, each sample was divided into 2 centrifuge tubes containing 5 ml ice-cold saline and processed further.

The repair inhibitors, ara A (Sigma) and ara C (Sigma) were dissolved in HBSS (Hanks balanced salts solution) at a concentration of 10

mmol/l. Ara C was sufficiently soluble in HBSS at this concentration, but 0.075 mol/l HCl was needed to dissolve the ara A. These stock solutions were routinely stored at 4 °C. The repair inhibitors were added to the cell suspensions 1 h before irradiation to allow for phosphorylation. The inhibitor concentration was kept the same at each stage of the experiment *i.e.* during the pre-irradiation incubation, irradiation and the repair time following irradiation.

4.2.2 *DNA unwinding assay and hydroxylapatite chromatography*

Lysing solution that contained 0.03 mol/l NaOH (pH 12) and either 0.15 mol/l or 1 mol/l NaCl, was freshly prepared just prior to each experiment and kept on ice. The cell samples (at 1.10^6 cells/sample) in ice-cold saline solution were centrifuged to a pellet and the supernatant aspirated. After vortexing, 0.5 ml of ice-cold lysing solution was forcefully added to each sample using a micropipette. Samples were left on ice undisturbed and under conditions of low light for 1 h. Subsequently, samples were neutralised by adding 1.1 ml of 0.02 mol/l Na_2HPO_4 and immediately sonicated for 6-10 s. After addition of 0.2 ml 0.85 mol/l SDS (25%), samples were vortexed and deep frozen (-20 °C) overnight.

After defrosting the samples were applied to small affinity columns comprising approximately 0.2 g of hydroxylapatite gel (Biorad, 50:50 mixture of Bio-Gel HTP and DNA grade HTP) held at 60 °C. Before and after sample application, the hydroxylapatite was washed with 2.5 ml of 0.0125 mol/l SPB (sodium phosphate buffer) at pH 6.8. Single-stranded DNA (ss) was then eluted with 2x 2.5 ml of 0.125 mol/l SPB and double-stranded DNA (ds) with 2x 2.5 ml 0.25 mol/l SPB. 0.3 ml 5 mol/l HCl and 5 ml scintillation cocktail (Optiphase MP, LKB) were added to each eluted sample and after vortexing the radioactivity per sample was determined by liquid scintillation counting.

The relative mass fraction of DNA in double-stranded form ($m_{ds}/m_{ds} + m_{ss}$) was obtained from the ratio $A_{ds}/(A_{ds} + A_{ss})$, where A_{ds} is the activity (dpm) measured in the double-stranded fraction and A_{ss} the activity measured in the single-stranded fraction. This relative mass fraction is indicative of the amount of undamaged or unbroken DNA. Dose-response curves were obtained by plotting the relative mass fraction against dose. In the repair experiments the relative mass fraction values were converted to 'dose' values using a dose-response curve. These dose values, expressed in Gy, reflect the amount of the X-ray damage remaining after the various repair times.

4.2.3 *Non-denaturing filter elution assay (pH 9.6)*

The neutral elution procedure is described in detail in Chapter 2 (section 2.2.2) and was performed at pH 9.6 and under conditions stipulated in section 2.4. The procedure followed in the repair experiments is described in detail in Chapter 3 (section 3.2.2).

4.2.4 *DNA synthesis assay*

Unlabelled EAT cells at a concentration of 5-6.10^5/ml were placed in glass bottles in a water bath and allowed to equilibrate to 37 °C. Ara A or ara C, in the form of a 10 mmol/l stock solution in HBSS, was added to various final concentrations. At chosen time intervals after the addition of inhibitor, a 1 ml aliquot of cells was removed to which 18.5 kBq of ^{3}H-TdR was rapidly added. After an incubation time of exactly 5 min at 37 °C, 5 ml of cold saline was forcefully added to the sample, which was then put on ice. This process was repeated at 10, 20, 40 and 60 minute intervals following the addition of ara A or ara C and control samples were run simultaneously. Once all the samples had been accumulated on ice, they were centrifuged and the supernatant aspirated. After vortexing the

pellets, 1 ml of 0.03 mol/l NaOH was added, followed by 1.5 ml of 0.61 mol/l TCA (trichloracetic acid) 10 min later. Samples were stored at 4 °C overnight to allow full precipitation of DNA.

The precipitated DNA was collected on to glass-fibre filters (Whatman), rinsed twice with ice-cold 0.31 mol/l TCA and finally with ice-cold absolute ethanol. 4 ml of scintillation cocktail (Optiphase MP, LKB) was added to the dried filters and radioactivity per filter determined by liquid scintillation counting.

The mean value of the ^{3}H-activity per filter at the various sampling times was determined for the control samples and this value was normalised to unity. The incorporation of ^{3}H-TdR in the presence of the inhibitor was then calculated relative to this normalised mean control value and plotted against the time elapsed between addition of the inhibitor and sampling.

4.3 Results

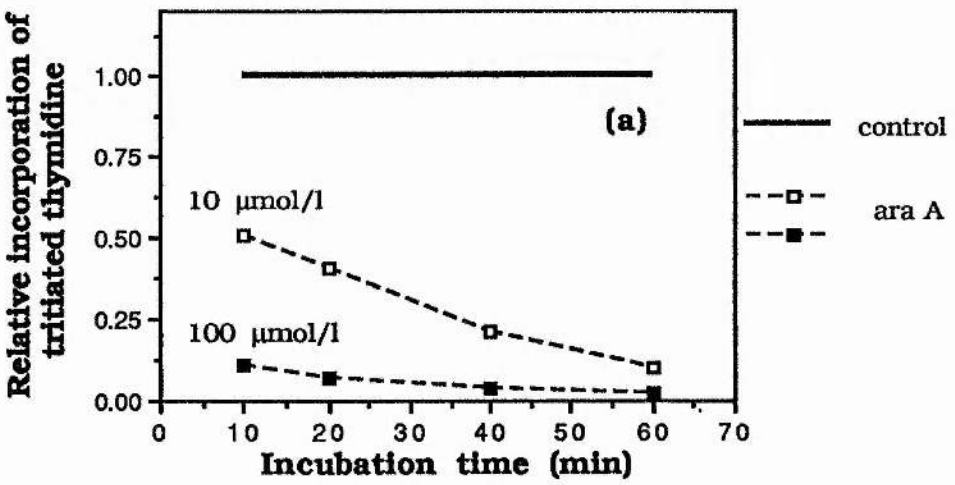
4.3.1 *Inhibition of DNA synthesis*

The inhibitory action of the nucleoside analogues ara A and ara C on DNA synthesis was tested by measuring the extent of ^{3}H-TdR (tritiated thymidine) incorporation into the DNA of EAT cells during a 5 min pulse. The amount of ^{3}H-activity incorporated into the DNA of the control samples of EAT cells during a 5 min pulse with 18.5 kBq ^{3}H-TdR and taken at various sampling times, is given in Table 4.1.

Incubation time (min)	^{3}H- dpm control$_1$	control$_2$
10	14 882	14 250
20	14 168	16 617
40	16 739	16 295
60	14 582	15 817
	(mean = 15 097)	(mean = 15 745)

Table 4.1 The amount of ^{3}H-activity incorporated into the DNA of EAT cells during a 5 min pulse with ^{3}H-TdR in the absence of the DNA synthesis inhibitors.

The incorporation of ^{3}H-activity in the presence of the DNA synthesis inhibitors was calculated relative to the mean values determined for the control samples as shown in Table 4.1, where the mean value of the controls was normalised to unity. The results of the DNA synthesis assay in the presence of the drugs are given in Fig. 4.1, where the cells were incubated in medium at 37 °C in the presence and absence of (a) ara A and (b) ara C.



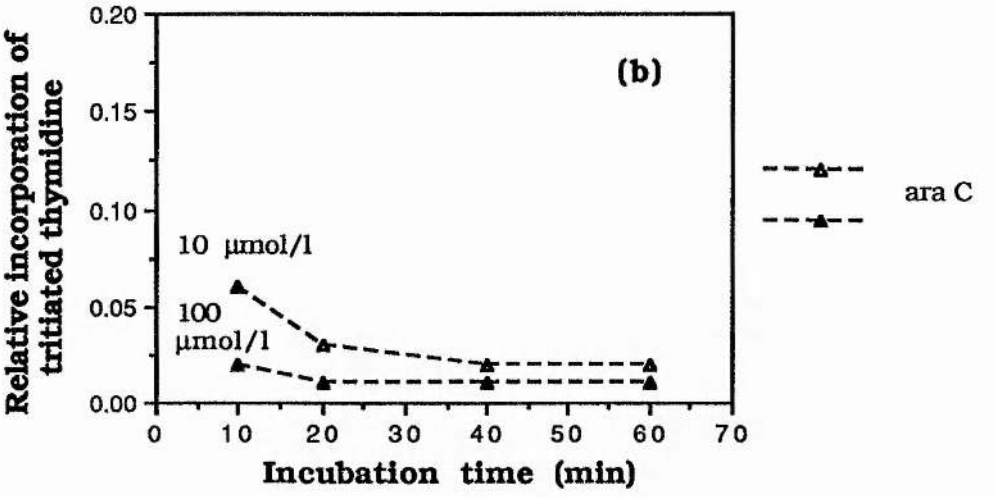


Figure 4.1 DNA synthesis assay: relative incorporation of ^{3}H-TdR into the DNA of EAT cells incubated at 37 ^{o}C in the presence and absence of (a) ara A and (b) ara C.

In the absence of the inhibitors the incorporation of ^{3}H-TdR into DNA was found to be approximately constant over the 1 h incubation period (Table 4.1). A strong decrease in incorporation was observed, that increased with incubation time, after the addition of 10 and 100 µmol/l ara A (Fig. 4.1a) or ara C (Fig. 4.1b). An ara A concentration of 100 µmol/l was sufficient to almost completely inhibit DNA synthesis, as measured by the incorporation of ^{3}H-TdR, after cells had been incubated in the presence of ara A for 1 h. Ara C was found to be more effective in inhibiting DNA synthesis, and 10 µmol/l was sufficient to completely inhibit ^{3}H-incorporation after a 20 min incubation interval. Since these were exponentially growing cells the above results imply that semi-conservative DNA synthesis can be virtually completely inhibited by either ara A (100 µmol/l) or ara C (10 µmol/l).

4.3.2 *DNA unwinding results*

A dose-response curve for EAT cells following X-irradiation obtained with the DNA unwinding method using 0.15 mol/l NaCl in the lysis solution, is shown in Fig. 4.2.

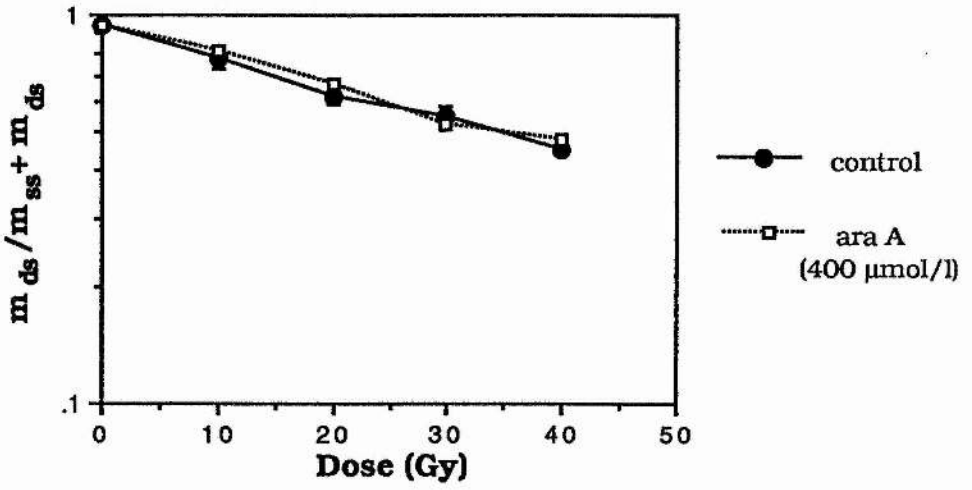


Figure 4.2 Dose-response curve for induction of DNA strand breaks in X-irradiated EAT cells in the presence and absence of 400 µmol/l ara A measured by the DNA unwinding method (pH 12). The frequency of strand breaks is inversely related to the logarithm of the function: ($m_{ds}/m_{ds}+m_{ss}$). Vertical bars represent the standard error of mean values.

The relative mass fraction ($m_{ds}/m_{ds}+m_{ss}$), which reflects the proportion of undamaged DNA (see section 4.2.2), is plotted against dose and can be seen to decrease in a linear manner with increasing dose. The response of cells which had been incubated in the presence of 400 µmol/l ara A for 1 hour before irradiation and irradiated in the presence of ara A (400 µmol/l) is shown by the dashed line. Clearly the presence of 400 µmol/l ara A did not affect the induction of DNA breaks by X-irradiation.

The DNA unwinding dose-response relationship of X-irradiated EAT cells, following lysis in high ionic strength (1 mol/l NaCl) alkaline solution (0.03 mol/l NaOH) is shown in Fig. 4.3.

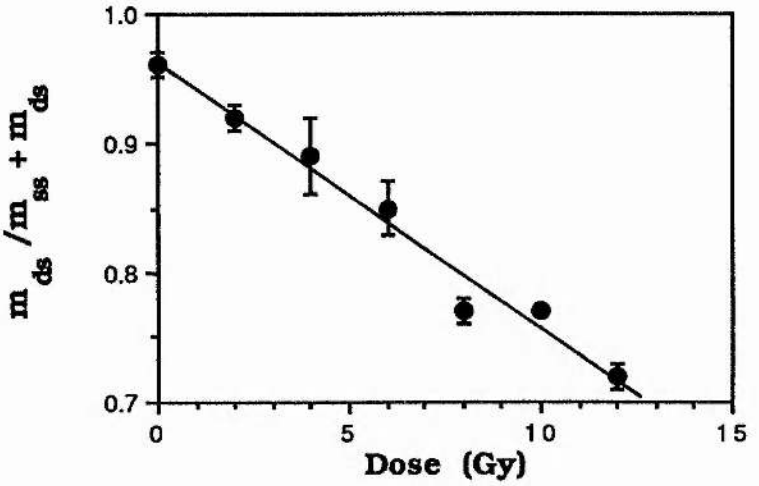


Figure 4.3 DNA unwinding dose-response of X-irradiated EAT cells following lysis in high ionic strength (1 mol/l NaCl) alkaline solution (pH 12). The data points are the mean of two experiments and the vertical bars the standard error of mean values.

Strand separation of the DNA under alkaline conditions (pH 12) is accelerated by high NaCl concentrations (Rydberg 1975). This substantially increases the sensitivity of the technique and thus facilitates dose-response measurements in the lower dose range of 0-12 Gy. The relative mass fraction ($m_{ds}/m_{ds} + m_{ss}$) shows a linear decrease with increasing dose and it was with the aid of this dose-response curve (Fig. 4.3) that the relative mass fraction values obtained in the repair experiments were converted to remaining damage values, to be expressed in Gy.

Using the DNA unwinding method, the kinetics of DNA repair of EAT cells were followed up to 1 h post-irradiation (8 Gy) in the presence and absence of ara A and ara C . These short-term repair results, assumed to reflect rapid ssb repair, are shown in Fig. 4.4.

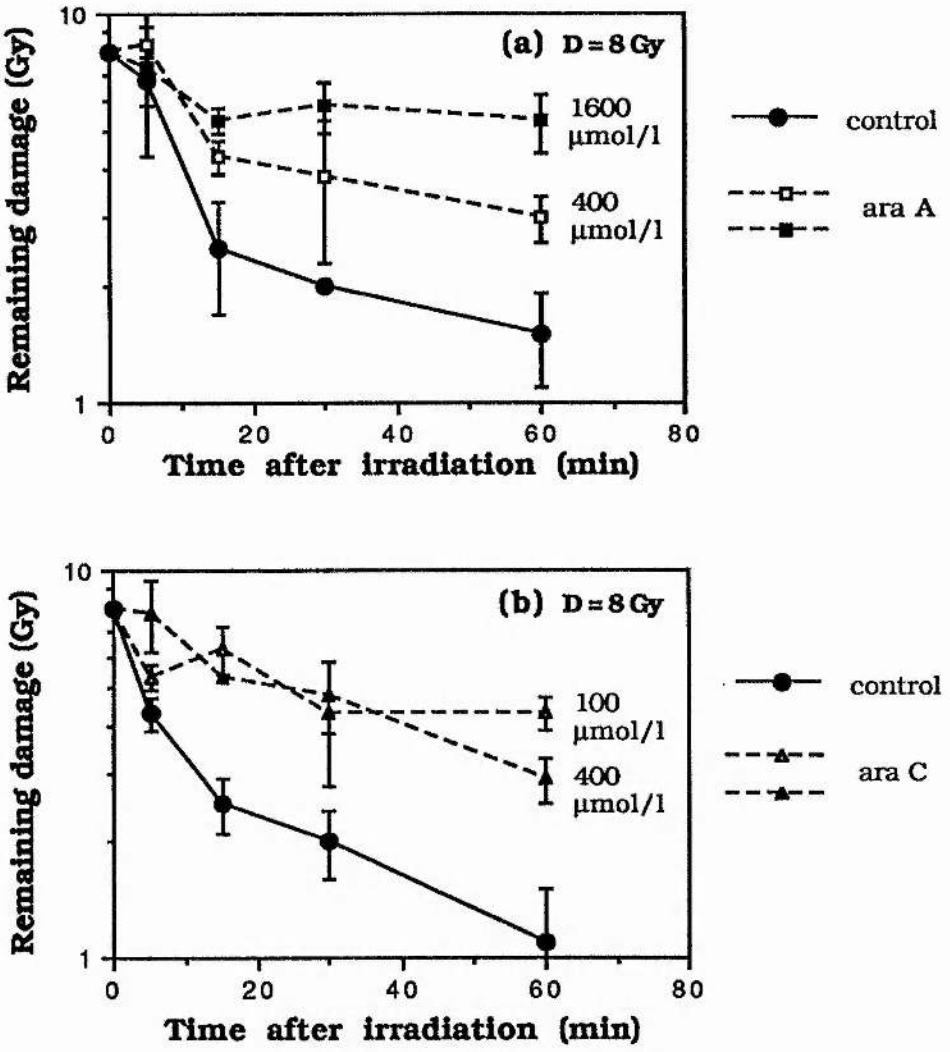
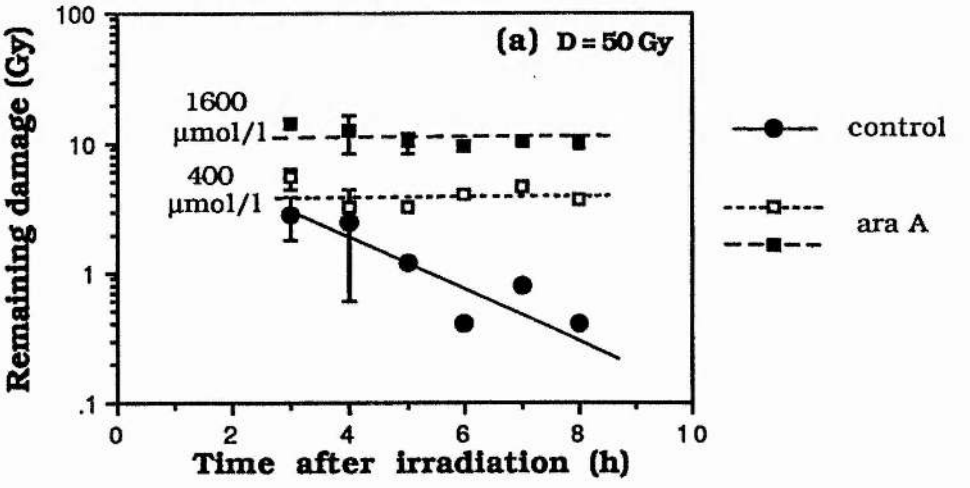


Figure 4.4 Kinetics of disappearance of remaining damage, interpreted as DNA ssb repair, as a function of time after X-ray exposure (8 Gy) in the presence and absence of (a) ara A and (b) ara C. Vertical bars represent standard error of mean values.

The control samples (in both (a) and (b)) show typical biphasic kinetics for ssb repair, as has been previously reported for EAT cells (Bryant *et al.* 1984). Lower concentrations of ara C were used, since ara C was found to inhibit DNA synthesis more effectively for the same concentration than ara A (see Fig. 4.1). From panel (a) it can be seen that ssb repair was inhibited in the presence of ara A and that the extent of inhibition was greater at 1600 µmol/l than at 400 µmol/l. It is obvious that even at the

exceedingly high concentration of 1600 μmol/l ara A, ssb repair was not completely inhibited. In the case of ara C (panel (b)) concentrations of 100 μmol/l and 400 μmol/l resulted in more or less the same extent of inhibition of ssb repair, but once again full inhibition of ssb repair was not achieved.

Using the DNA unwinding method, the repair of EAT cells after exposure to 50 Gy of X-rays was followed from 2 h to 8 h post-irradiation. These long-term repair experiments, assumed to reflect dsb repair, were performed in the presence and absence of (a) 400 and 1600 μmol/l ara A and (b) 100 and 400 μmol/l ara C and the results are presented in Fig.4.5.



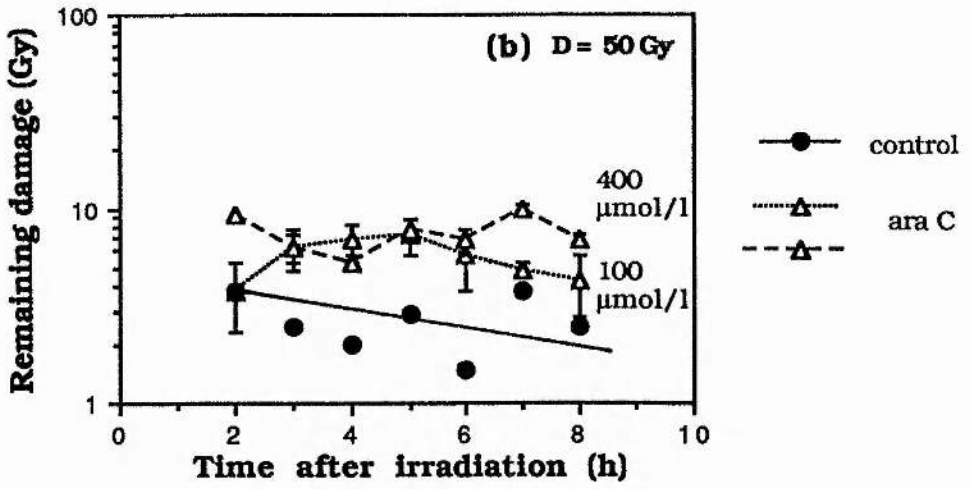


Figure 4.5 Kinetics of disappearance of remaining damage, interpreted as DNA dsb repair, as a function of time after X-ray exposure (50 Gy) in the presence and absence of (a) ara A and (b) ara C. Vertical bars represent standard error of mean values.

A disappearance of remaining damage (Gy) with time was observed in the control samples (panel (a)), which followed approximate first-order kinetics with a $t_{1/2}$ of some 2-3 h. This kinetic is in agreement with previous DNA unwinding repair data for EAT cells (Bryant and Blöcher 1980). Cells that were incubated in the presence of 400 µmol/l ara A show a constant level of remaining damage, which implies that no dsb repair has taken place and this would seem to indicate that dsb repair is completely inhibited at this concentration of ara A. The data at 1600 µmol/l ara A also show no repair, but reveal a higher level of remaining damage.

The disappearance of remaining damage (Gy) with time for the control samples in (b) show slower kinetics than measured in (a), with a $t_{1/2}$ of 4-5 h. The reason for this is not understood (but could have been due to unfavourable repair conditions on the day of the experiment). The cell samples that had been incubated in the presence of 100 or 400 µmol/l ara C show slightly higher and more or less constant levels of

remaining damage over the 8 h incubation period, which would also signify complete inhibition of dsb repair. The data obtained at the two concentrations (100 and 400 μmol/l) showed no significant difference, unlike the results obtained with ara A (panel (a)).

4.3.3 *Non-denaturing filter elution (pH 9.6) results*

Dose-response curves as measured by non-denaturing filter elution (pH 9.6), assumed to reflect dsb induction, are shown in Fig. 4.6 (background elution values have been subtracted).

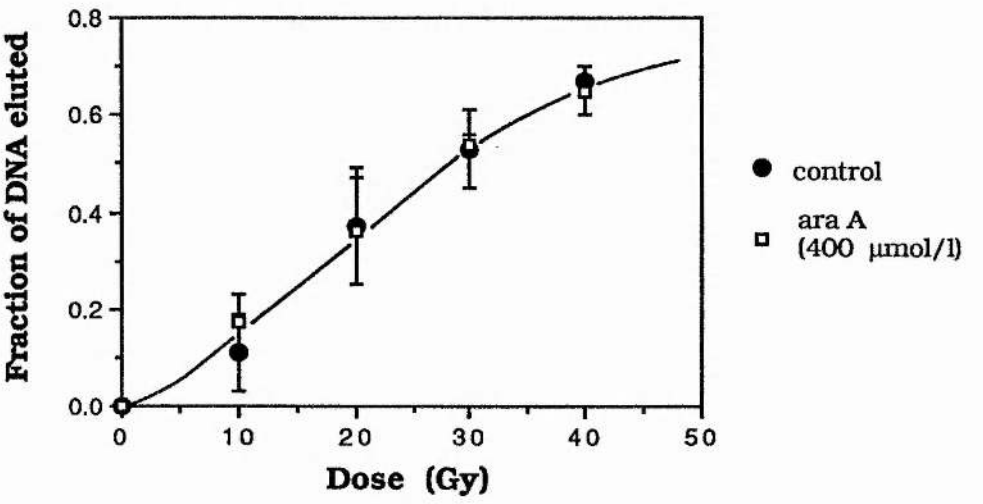


Figure 4.6 The dose-response for X-irradiated EAT cells, in the presence and absence of ara A, measured by non-denaturing filter elution (pH 9.6). The fraction of DNA eluted at 16 h was used and the points represent the mean of 2 experiments and the vertical bars the standard error of mean values.

The sigmoidal fitting is based on previous neutral elution experiments (see Fig. 3.4). Clearly the presence of 400 μmol/l ara A did not affect the induction of dsb by X-rays. It is interesting to note that the dose-response curve obtained for EAT cells is very similar to that obtained for CHO cells (see Fig. 3.4).

The repair after a dose of 30 Gy was followed up to 3 h post-irradiation using the neutral elution assay (pH 9.6) and the results obtained in the presence and absence of ara A are shown in Fig. 4.7.

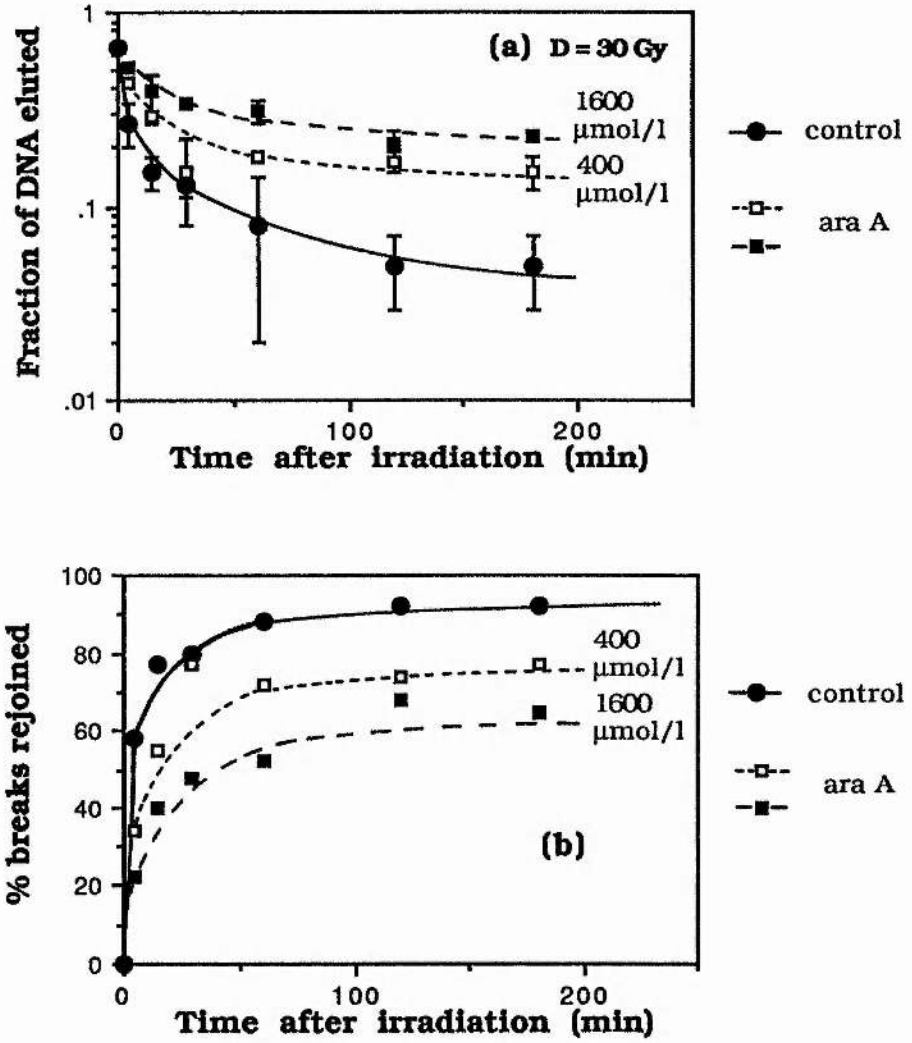
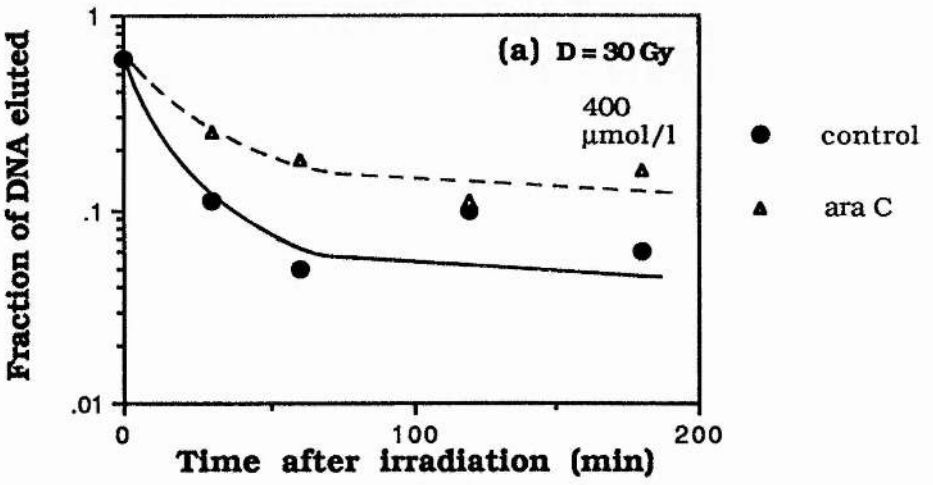


Figure 4.7 Kinetics of disappearance of DNA strand breaks measured by non-denaturing filter elution (pH 9.6), as a function of incubation time after X-ray exposure (30 Gy) in the presence and absence of ara A. The data points represent the mean of 3 experiments and the vertical bars represent standard error of mean values. In (b) the data from (a) is calculated as % breaks rejoined.

The decrease in the fraction of DNA eluted with time (Fig. 4.7a) indicates that rejoining of the DNA had occurred. The data suggest biphasic repair kinetics and estimates of the $t_{1/2}$ of the rapid and slow repair

components for the control samples were 6 min and 114 min respectively. The cells that had been incubated in the presence of 400 µmol/l ara A show marginally less repair and the 1600 µmol/l samples less again. The data from (a) has been plotted as % breaks rejoined in (b), as described in section 3.2.2. From (b) it can be seen that for the control data approximately 90 % of the breaks were rejoined during the 180 min period following irradiation, whereas 75 % of the lesions were repaired in the presence of 400 µmol/l ara A and 60 % in the presence of 1600 µmol/l.

The results of the repair assay, using neutral elution (pH 9.6) in the presence and absence of ara C are given in Fig. 4.8.



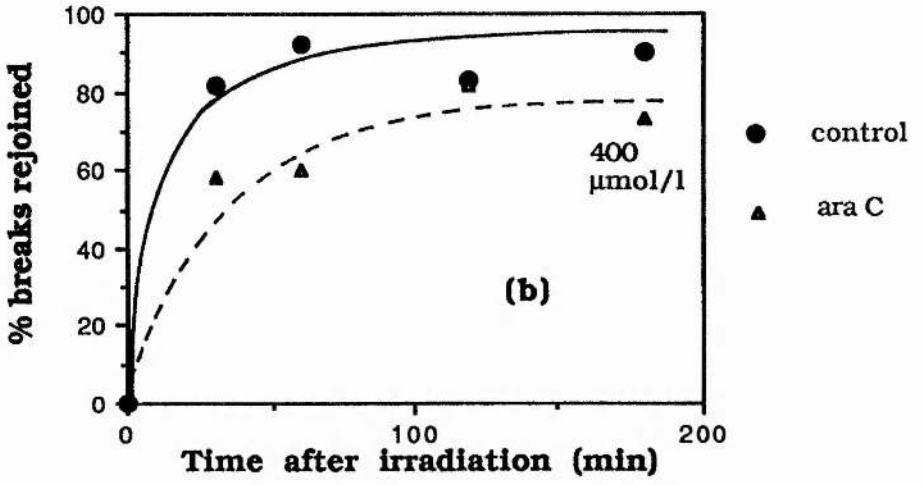


Figure 4.8 Kinetics of disappearance of DNA strand breaks measured by non-denaturing filter elution (pH 9.6), as a function of incubation time after X-ray exposure (30 Gy) in the presence and absence of ara C. In (b) the data from (a) is calculated as % breaks rejoined.

The control data, although not as detailed as in Fig. 4.7, also reflects biphasic repair kinetics. The samples that had been incubated with 400 µmol/l ara C show marginally less repair. From (b) it can be seen that 90 % of the breaks had rejoined during the 180 min post-irradiation incubation period for the control samples and approximately 78 % of the lesions were repaired in the presence of 400 µmol/l ara C.

4.4 Discussion

It has been shown that the presence of both ara A and ara C result in strong inhibition of DNA synthesis in EAT cells (Fig. 4.1). The literature would suggest that this inhibition is due to the inhibitory action of these nucleoside analogues on DNA polymerase, especially polymerase α (Müller *et al.* 1977; Okura and Yoshida 1978; Stammberger *et al.* 1989) which is generally accepted as the polymerase responsible for DNA

replication in the nucleus (reviewed in Miller and Chinault 1982). My data (Fig. 4.1) would suggest that ara C inhibits DNA synthesis more effectively than ara A, since complete inhibition by ara C was achieved at one tenth of the concentration required for ara A.

The short-term repair experiments performed with the DNA unwinding technique (Fig. 4.4), show the inhibitory effect of ara A and ara C on what is assumed to be ssb repair. These results are in agreement with the results of Bryant and Blöcher (1982), who found significant inhibition of ssb repair by ara A in EAT cells for concentrations greater than 500 µmol/l. The results from Fig. 4.4 suggest that the inhibition of ssb repair increases with increasing ara A concentration, whereas the ara C concentration did not seem to affect the extent of inhibition.

A similar tendency is observed in the long-term repair experiments using the DNA unwinding method, which is assumed to reflect dsb repair (Fig. 4.5). There seems to be a concentration effect with ara A but not with ara C. Full inhibition of dsb repair was obtained at 400 µmol/l ara A and at 100 µmol/l ara C. The elevated level of residual damage measured at 1600 µmol/l ara A could possibly be due to the extensive inhibition of ssb repair, as observed in Fig. 4.4 (b), but the effect of complete deregulation of cell metabolism at this high ara A concentration cannot be ruled out.

The results presented in Fig. 4.5 essentially confirm those obtained by Bryant and Blöcher (1982) and Iliakis and Bryant (1983), who using the DNA unwinding method, found that these drugs inhibit the repair of dsb. Bryant and Blöcher (1982) measured the same inhibitory effect with the neutral velocity sedimentation assay, which verifies that DNA unwinding does in fact detect dsb repair under the conditions mentioned above. Iliakis and Bryant (1983), using equal concentrations of ara A and

ara C, determined that ara C resulted in greater inhibition of dsb repair but the data shown in Fig. 4.5 does not substantiate this.

In contrast to these results obtained with DNA unwinding, the neutral elution assay at pH 9.6 (Figs. 4.7 and 4.8) shows only limited inhibition of dsb repair in the presence of ara A and ara C. From Fig. 4.7 (b) it can be seen that 80 % of repair (when normalised to the control value of 90 %) occurred in the presence of 400 μmol/l ara A, and about 67 % repair at 1600 μmol/l ara A when compared with controls. Similarly, Fig. 4.8 shows limited inhibition of repair in the presence of ara C, which corresponds to approximately 80 % of rejoining when normalised to the control value of 90 %.

The actual mechanism/s of dsb repair are as yet unknown and it is therefore difficult to reconcile the effect of ara A and ara C on dsb repair with the inhibitory action of the drugs on the DNA polymerases α or β. It should also be noted that the underlying mechanism by which these drugs inhibit DNA synthesis has not been fully elucidated. This investigation of the neutral elution assay using ara A and ara C is therefore solely based on the empirical observation of the drugs' inhibitory action on dsb repair of Bryant and Blöcher (1982) and Iliakis and Bryant (1983).

Due to the different experimental conditions used in the two assays presented here, it was not possible to draw a direct comparison between the actual numbers of unrepaired dsb. The aim was rather to compare relative rates and extent of repair in the presence of the inhibitors, ara A and ara C, and thereby to determine if non-denaturing filter elution (thought to detect dsb) revealed the same extent of inhibition of dsb repair. The results presented here with ara A and ara C therefore suggest that neutral elution (pH 9.6) might detect a lesion that is different from that measured by DNA unwinding or neutral velocity sedimentation. The fact that only limited inhibition of dsb repair was observed in the

presence of the DNA synthesis inhibitors, indicates the possibility that non-denaturing filter elution may detect a specific type of dsb which is repaired under conditions where DNA polymerization is drastically reduced.

The finding that non-denaturing filter elution and DNA unwinding are not detecting the same type of lesion, suggests the existence of more than one type of dsb. These possibly disparate dsb are distinguished on the basis of the differential requirement for DNA polymerization for repair. The results presented in this chapter would seem to indicate that the rejoining mechanisms in question are (a) a simple ligation process which does not involve DNA polymerases, and (b) a, as yet unknown, mechanism that has an absolute requirement for DNA polymerization. These results would thus suggest that neutral elution detects a type of dsb which can be rejoined by simple ligation whereas DNA unwinding and velocity sedimentation monitor dsb which have a prerequisite of DNA polymerization for repair.

Chapter 5

EXPERIMENTS USING RESTRICTION ENDONUCLEASES

5.1 Introduction

5.1.1 Restriction endonucleases (RE)

5.1.2 Electroporation

5.2 Materials and methods

5.2.1 Cell culture and labelling

5.2.2 Purification of the RE

5.2.3 Electroporation and subsequent incubation

5.2.4 Non-denaturing filter elution assay (pH 9.6)

5.3 Results

5.4 Discussion

5.1 Introduction

The aims of this work were two-fold; firstly to determine whether the non-denaturing filter elution technique detects dsb in cellular DNA and secondly to follow the induction and repair of the dsb induced by restriction endonucleases (RE). RE that generate dsb with various end-structures were used and in so doing it was hoped that they would be of help in solving the question as to the nature of the breaks detected by non-denaturing filter elution.

5.1.1 *Restriction endonucleases (RE)*

Restriction endonucleases (RE) are enzymes, isolated from a variety of bacteria, which can degrade DNA by cleavage of both strands of the DNA at specific recognition sequences (Roberts 1987). As a result of the ability of RE to induce dsb in mammalian DNA they have been implemented to model the dsb-inducing action of radiation (Bryant 1984). The advantage of using RE is that, unlike radiation which induces a range of DNA lesions (ssb, dsb, cross-links, base damage), these enzymes generate solely dsb. RE thus allows a study of the biochemical and cytogenetic consequences of DNA dsb, without the interference of other lesions.

The RE used in this study, *Pvu* II, *Bam* H1 and *Eco* R1 (Northumberland Biological Laboratories), have the property of recognising a specific 6 base sequence. The site at which the dsb is generated is fixed with respect to the recognition sequence and is unique to each RE:

Pvu II 5' --CAG▼CTG-- 3'
3' --GTC▲GAC-- 5'

Bam H1 5' --G▼GATCC-- 3'
3' --CCTAG▲G-- 5'

Eco R1 5' --G▼AATTC-- 3'
3' --CTTAA▲G-- 5'

The cutting frequencies of these enzymes in mammalian DNA are partly determined by the frequency of the recognition sites in the genome. A limitation on the number of available cutting sites will be the state of condensation of DNA. From a model proposed by Bishop *et al.* (1983) *Pvu* II is expected theoretically to cut mammalian DNA every 2900-3200 bases, *Bam* H1 once every 5500-7000 bases and *Eco* R1 every 3000 bases (Bryant 1988; Winegar and Preston 1988).

It can be seen from the incision sites within the recognition sequences shown above, that *Pvu* II produces double-strand breaks with blunt-ended termini whereas *Bam* H1 and *Eco* R1 produce cohesive-ended dsb with a 4 base overlap. This difference in the action of the above RE was exploited to investigate whether the ability of non-denaturing filter elution to detect dsb is affected by the end structure of the breaks.

Up till now treatment of living cells with RE has mainly been used in cytogenetic investigations where it was shown that CA are induced by dsb in DNA of cells (Bryant 1984; Natarajan and Obe 1984; Obe *et al.* 1985; Natarajan *et al.* 1985; Obe and Winkel 1985; Gustavino *et al.* 1986; Winegar and Preston 1988). Other end-points such as mutation (Obe *et al.* 1986), cell lethality (Bryant 1985) and oncogenic transformation (Bryant and Riches 1990) have since been studied. Only two attempts have been made to measure frequencies of restriction dsb using

biochemical assays *viz.* by Bryant (1984) and Natarajan *et al.* (1985), and no studies have as yet been able to address the question of the kinetics of repair of RE-generated breaks.

Although the mechanisms of the repair of radiation- or RE-induced breaks are not known, it is thought unlikely that the repair is exactly the same in both cases (Bryant 1988). This is due to the fact that RE breaks have 'clean' 3'-hydroxyl and 5'-phosphoryl termini which may simply require ligation to be rejoined, whereas radiation-induced dsb are thought likely to have 'dirty' ends which require enzymatic modification before repair can take place (Henner *et al.* 1982).

A major limitation encountered in the use of RE in modelling radiation effects, has been the less than optimal methods by which the RE have been introduced into the cells. These methods include the use of inactivated Sendai virus (Bryant 1984), the 'pellet' method of Obe *et al.* (1985) and treatment with hypertonic or hypotonic shock (Winegar and Preston 1988). A major inconsistency which has led to considerable controversy centres around the relative effectiveness of RE that generate cohesive-ended dsb in causing chromosomal aberrations. Some workers have found these enzymes to be less effective than RE that induce blunt-ended dsb (Bryant 1984; Natarajan and Obe 1984; Bryant *et al.* 1987), whereas others have found them to be equally effective (*e.g.* Gustavino *et al.* 1986; Winegar and Preston 1988). These inconsistencies between the results obtained by various workers have been attributed to the different techniques employed to permeabilise the cells to RE (Bryant 1988; Bryant and Christie 1989).

5.1.2 *Electroporation*

Recently Winegar *et al.* (1989a) have shown that the technique of electroporation is a rapid and efficient method for introducing RE into

mammalian cells. They found that electroporation gave more reliable results than the above mentioned techniques in that the cell population was evenly permeabilised.

The basic principle of the electroporation technique is based on the fact that the cell membrane is unable to pass electric current and therefore, when it is subjected to a high-voltage DC electric pulse, thinning and reversible breakdown of the membrane occurs in localised areas (Knight 1981; Potter 1988). The resulting 'pores' in the membrane will reseal provided the magnitude or duration of the electric pulse does not exceed critical limits beyond which the cell will be irreversibly damaged (Shigekawa and Dower 1988). The diameter, lifetime, numbers and location of these pores have actually been visualized using low light level video microscopy (Sowers and Lieber 1986) and pulsed-laser fluorescence microscopy (Kinosita *et al.* 1988).

The most important parameters in determining the success of the electroporation treatment are the maximum voltage of the electric field, the duration of the pulse and the composition of the electroporation buffer. These parameters need to be optimised for each cell type (Potter 1988, Andreason and Evans 1988). Under the correct conditions the size and lifetime of the transient pores will allow the uptake of large molecules such as enzymes (Andreason and Evans 1988) and even larger molecules up to the size of DNA (Chu *et al.* 1987).

A Bethesda Research Laboratories (BRL) Cell-Porator electroporation system and disposable electroporation chambers were used in this study. This Cell-Porator uses a capacitor discharge to deliver a exponentially decaying pulse, the duration of which is determined by the capacitance and resistance setting (the greater the capacitance or resistance the longer the pulse), and by the conductivity of the solution (the higher the conductivity the shorter the pulse). The field-strength

(E) is determined by the voltage setting (V) : $E = {}^{V}/_{d}$, where d is the distance between the electrodes (d=0.4 cm for the disposable electroporation chambers used here).

In this study the electroporation technique was employed to permeabilise CHO cells to RE and the conditions, found to be optimal for CHO cells, of Winegar *et al.* (1989a) were used. The only difference was that the cells were electroporated in MEM rather than HEPES-buffered saline (Winegar *et al.* 1989a), as preliminary control experiments showed that cell survival was higher after electroporation in MEM.

5.2 Materials and methods

5.2.1 *Cell culture and labelling*

Asynchronous populations of exponentially growing CHO K1 cells were used and the cell culture and labelling procedures were as described in sections 2.2.1 and 2.3.1. Briefly, 75 cm^2 plastic tissue culture flasks were seeded with 1.10^6 cells in 10 ml MEM and labelled with 3.7 kBq/ml ^{3}H-TdR for 48 h.

5.2.2 *Purification of the RE*

Restriction endonucleases were routinely kept at - 20 °C in a storage buffer (SB) containing 50 % glycerol. In preliminary experiments, the storage buffer alone was found to have a detrimental effect on electroporated cells in that increased elution of the DNA was observed. It was therefore decided to purify the enzymes free of storage buffer. Purification was achieved by ultrafiltration with Amicon-10 filters (Bryant and Christie 1989), which are 'molecular sieve' type filters with a

10 000 dalton cut off point (the molecular mass of RE are in the range of 20-60 Kd).

Amicon-10 filters were sterilised by rinsing repeatedly with 70 % alcohol and finally with sterile distilled water. The filters were saturated with bovine serum albumin (BSA) protein to prevent the loss of the RE during the recovery step by adsorption to the filter, and kept on ice. The RE, diluted in 1 ml HBSS was applied to the filter and subjected to centrifugation at 8 K rpm for 1 h at 2 °C. The dilution and centrifugation was repeated before recovery of the RE in approximately 50-60 µl HBSS. The RE was then prepared to a final concentration of 10 units/µl in HBSS containing 1 % BSA and 6 mmol/l $MgCl_2$ and stored on ice.

5.2.3 *Electroporation and subsequent incubation*

After trypsinization, cells were resuspended in MEM to give a final concentration of 1.10^6 cell/ml. Electroporation was performed in MEM at ambient temperature using a BRL Cell-Porator. Appropriate amounts of purified RE were mixed with 1 ml of cell suspension in an Eppendorf tube before transfer into a disposable electroporation chamber. The electroporation conditions of Winegar *et al.* (1989a) were used: field-strength 650 V/cm, capacitance 1600 µF and the electroporator set at low resistance.

Following electroporation treatment, the samples were either poured directly into petri dishes together with 3 ml MEM used to rinse the electroporation chamber; or the samples plus the 3 ml rinse were poured into V-tubes, centrifuged, the supernatant aspirated and after resuspension in 4 ml MEM, poured into petri dishes. In the latter case, any enzyme present in the medium after electroporation treatment was removed whereas in the former it was not.

One of two procedures was followed for the incubation in a humidified, CO_2 incubator at 37 °C and subsequent collection of the samples: (1) Cells were incubated in non-tissue culture petri dishes for various times, resuspended by pipetting and the total sample volume was then introduced into ice-cold PBS for the neutral elution step. Or (2) cells were incubated in tissue culture petri dishes for various times and subsequently removed by trypsinization. In this case the medium and the trypsin washes were discarded and therefore only the cells that had remained attached to the dishes were selected for the neutral elution step.

In the case of the ara A experiments (described in detail in Chapter 4, section 4.2.1), ara A was added to the flasks to a final concentration of 400 µmol/l, approximately 1 h before trypsinization to allow for phosphorylation, after which the cells were resuspended, electroporated and plated in MEM containing 400 µmol/l ara A.

The treatments were staggered such that all samples were collected at a common time point and after resuspension in MEM the samples were introduced into 10 ml ice-cold PBS in the reservoirs of the filter elution apparatus.

5.2.4 *Non-denaturing filter elution assay (pH 9.6)*

The neutral elution procedure as described in section 2.2.2 was performed at pH 9.6 and under the lysis and eluting conditions stipulated in section 2.4. The only difference was that the PBS had to be pumped through to load the cells on to the 2 µm polycarbonate filters, since the electroporated samples had a tendency to block the filters when run under gravity, presumably due to remaining cell debris in the medium.

5.3 Results

Preliminary attempts to measure dsb by non-denaturing filter elution in CHO cells treated with RE, in which the cells were permeabilised using inactivated Sendai virus, were of limited success. Similar results using inactivated Sendai virus as a permeabilising agent had been reported with the DNA unwinding technique (Bryant 1984). In contrast, electroporation was found to be an effective means of permeabilising CHO cells to RE and the resulting dsb could be detected by neutral elution if enough time was allowed to elapse after treatment. The results of one of the earliest experiments performed are given in Fig. 5.1. Electroporated CHO cells were treated with 400 units/ml unpurified *Pvu* II (in 4 µl SB) or 4 µl SB alone and the resulting elution values, assumed to reflect levels of dsb, were measured at short incubation times (0-1 h).

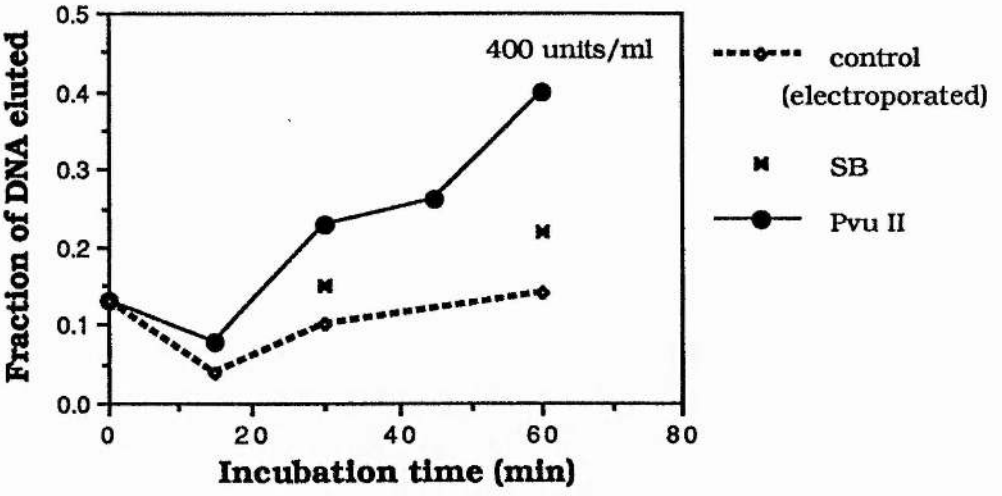


Figure 5.1 The levels of dsb in electroporated CHO cells treated with 400 units/ml *Pvu* II in storage buffer (SB) or 4µl SB, as measured by neutral elution (pH 9.6) up to 1 h post-treatment.

Clearly the numbers of dsb in the electroporated control cells and *Pvu* II treated cells increased over the incubation time, with the *Pvu* II treated

cells showing a larger increase. Electroporated cells that had been treated with 4 µl storage buffer alone (as denoted by the two SB points in Fig. 5.1), resulted in a significant level of damage and therefore it was decided to use purified RE in all subsequent experiments. The activity of *Pvu* II was found to be higher after purification, as monitored by the comparative efficiency of equal concentrations of purified and unpurified enzyme to linearise a specified amount of plasmid pBR322 DNA (Bryant and Moses, in preparation).

Fig. 5.2 shows the levels of dsb in electroporated CHO cells after treatment with 200 units/ml purified *Pvu* II and as measured up to 3 h post-treatment.

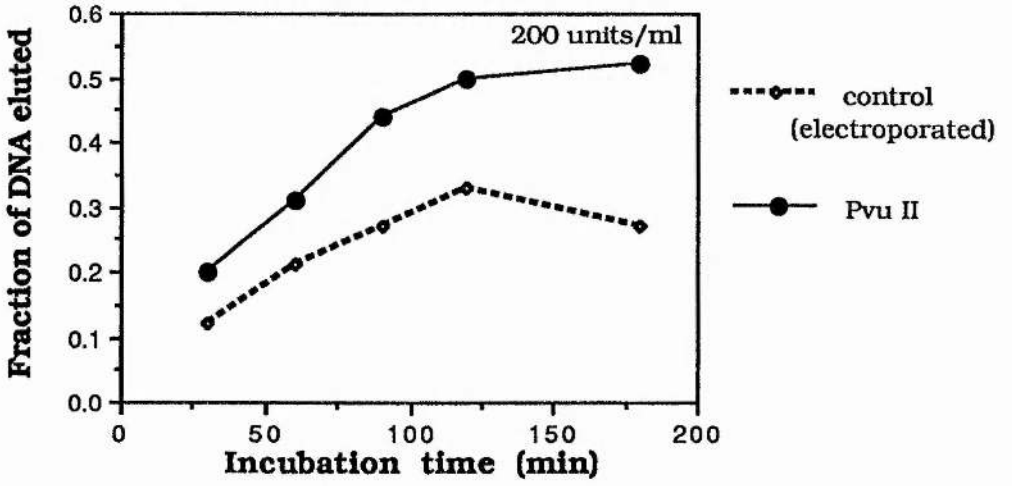


Figure 5.2 The levels of dsb in electroporated CHO cells treated with 200 units/ml purified *Pvu* II, as measured by neutral elution (pH 9.6) up to 3 h post-treatment.

It is evident that the levels of dsb are still increasing up to 3 h after treatment in the *Pvu* II treated samples, with a possible levelling off in the electroporated control samples. It was decided to extend the incubation times even further in an attempt to find a maxima in the induction curve. Fig. 5.3 shows the results of electroporated CHO cells

treated with 200 units/ml purified *Pvu* II and *Bam* H1 with incubation times of up to 6 h post-treatment.

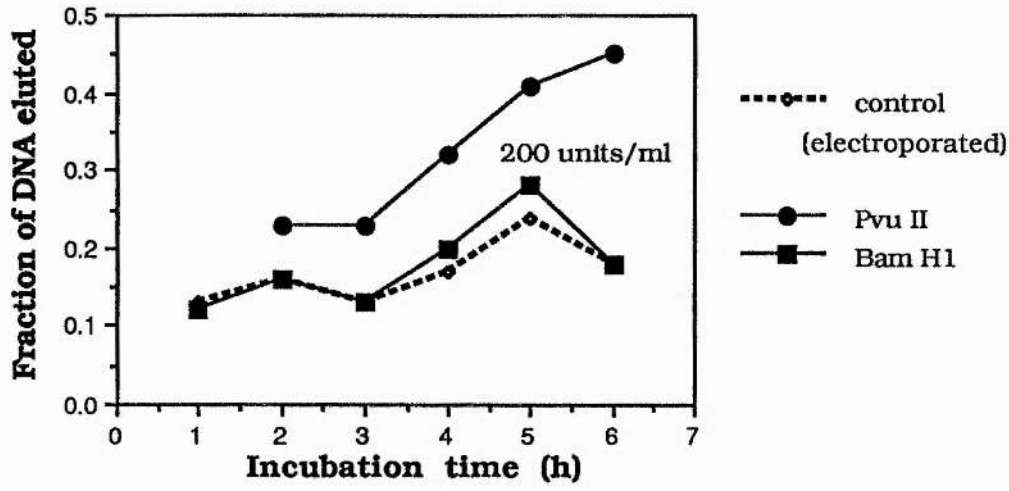


Figure 5.3 The levels of dsb in electroporated CHO cells treated with 200 units/ml *Pvu* II and *Bam* H1 (purified), as measured by neutral elution (pH 9.6) up to 6 h post-treatment.

Clearly the levels of *Pvu* II induced dsb were still increasing at 6 h post-treatment, whereas values for the *Bam* H1 and electroporated control samples were decreasing at 6 h. The levels of dsb in the *Bam* H1 treated cells were approximately the same as for the electroporated control samples.

A dose-effect curve for electroporated CHO cells treated with *Pvu* II and *Bam* H1 as measured by neutral elution (pH 9.6) is shown in Fig. 5.4. The levels of dsb were measured after 6 h incubation at 37 °C. The electroporation treatment alone (shown as control data) is represented by the dashed line and was extended beyond the zero-point on the x-axis to denote that the RE data points are in fact superimposed on this background level of dsb.

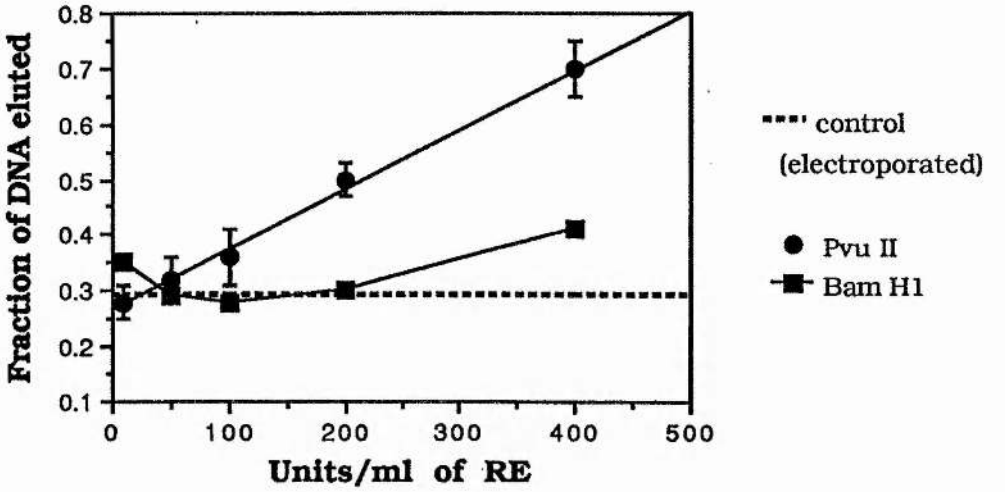


Figure 5.4 A dose-effect curve for electroporated CHO cells treated with *Pvu* II and *Bam* H1 (purified) as measured by neutral elution (pH 9.6) after 6 h post-treatment incubation. The data represent the mean of 2 experiments and the vertical bars the standard error of mean values. The dashed line represents the electroporated controls.

The linear increase in the numbers of dsb (fraction of DNA eluted) with *Pvu* II concentration confirms that electroporation is allowing entry of the RE into the cells. The *Bam* H1 data represent the results of a single experiment and it can be seen that *Bam* H1 is considerably less effective in inducing measurable dsb than *Pvu* II. Only at a concentration of 400 units/ml of *Bam* H1 were any dsb observed above the electroporated control value.

In Fig. 5.5 the elution profiles at pH 9.6 obtained for X-irradiated CHO cells and RE treated electroporated CHO cells treated with *Pvu* II are compared.

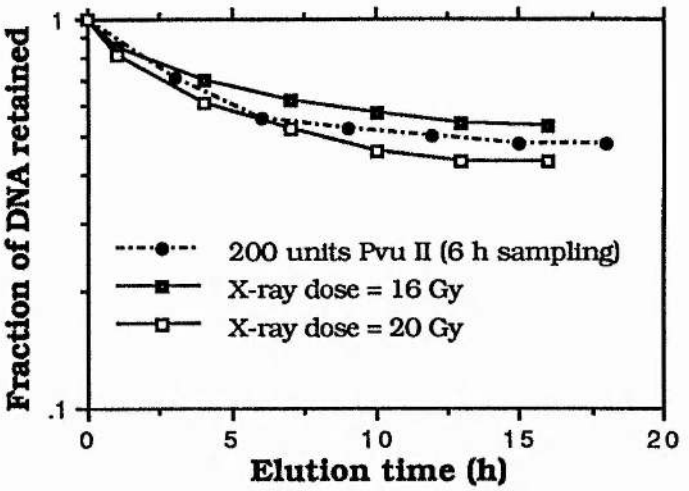


Figure 5.5 Comparison of the elution profiles of X-irradiated CHO cells and electroporated CHO cells treated with 200 units/ml *Pvu* II obtained at pH 9.6.

Cells that had been treated with 200 units/ml *Pvu* II gave elution profiles that corresponded to the profiles of cells that had received between 16 and 20 Gy of X-rays. The shape of these elution profiles were very similar which would suggest that the eluted DNA fragment size distributions were independent of the dsb inducing agent *i.e.* X-rays or RE.

From Figs. 5.1-5.4 it can be seen that the electroporation treatment alone (shown as control data) caused a significant amount of damage and therefore a treatment protocol was sought that would reduce this 'background' level of breaks. In Fig. 5.6 various protocols concerning treatment with *Pvu* II are compared and the levels of dsb were measured as a function of incubation time after electroporation treatment for up to 12 h. A *Pvu* II concentration of 200 units/ml was used throughout. In Fig. 5.6 (a) the electroporated cells were incubated in non-tissue culture petri dishes and the **total** cell population was harvested (by pipetting) for the neutral elution assay. In Fig. 5.6 (b) the cells were incubated in tissue culture petri dishes and only those cells that had remained **attached**

during the ensuing incubation time were harvested for the neutral elution assay *i.e.* only the **adhering** cells were selected for the dsb assay.

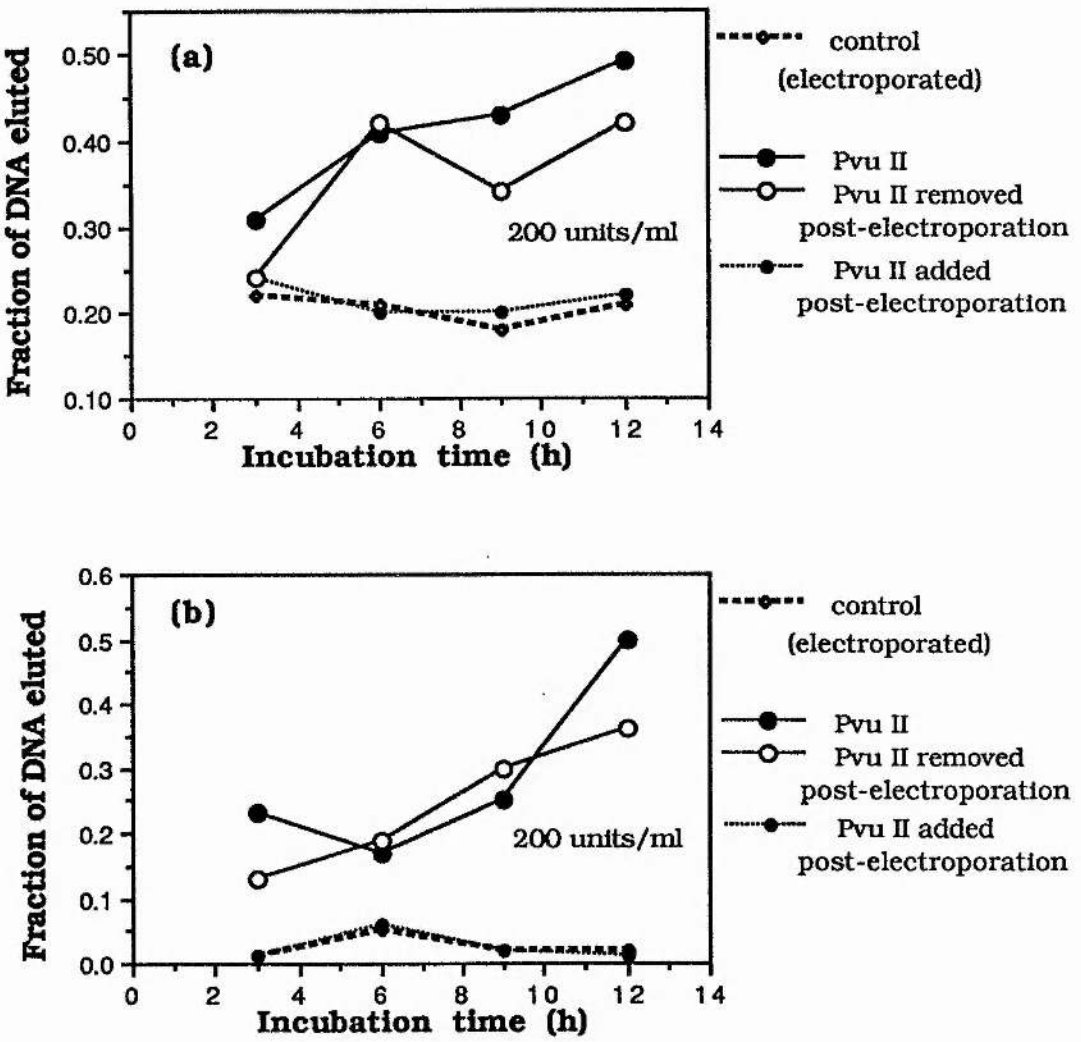


Figure 5.6 The levels of dsb as measured by neutral elution (pH 9.6) in electroporated CHO cells treated with 200 units/ml *Pvu* II, comparing various protocols concerning the treatment with *Pvu* II, subsequent plating and harvesting of the samples. Cells in (a) were incubated in non-tissue culture petri dishes and in (b) in tissue culture petri dishes.

It can be seen that by selecting only the attached cells ((b) versus (a)) that the background level of dsb due to the electroporation treatment was drastically reduced. The elevated levels of dsb in the control samples (in (a)) is probably due to a proportion of the cells that had been lethally

damaged by the electric pulse and indeed these could be seen in the medium above attached cells in the tissue culture dishes.

Pvu II which was added to the cell suspension immediately after electroporation (denoted by the small circles and the dashed line in Fig. 5.6) show the same low level of dsb induced with time as the electroporated controls, which implies that Pvu II is not gaining entry into the cell after the duration of the pulse.

The large closed circles, in Figs. 5.6(a) and 5.6(b), denote the cells that had been poured directly into the petri dishes immediately following the electroporation treatment and that were therefore incubated in medium that still contained *Pvu* II. The open circles denote the samples that were centrifuged, the supernatant removed and replaced with fresh medium and which were therefore incubated in the absence of extracellular *Pvu* II. Clearly the presence of *Pvu* II in the medium during the incubation period (large closed circles as compared to the large open circles) did not significantly increase the number of dsb generated, which indicates that the pores had effectively resealed and had prevented further uptake of the RE. This is further evidence that the lifetime of the pores in the cell membrane is extremely short, and this is also supported by the findings of Sowers and Lieber (1986) who estimated the lifetime of the pores in electroporated erythrocyte ghosts to be in the range of 200 ms.

It should be noted that the difference between the frequencies of dsb induced by *Pvu* II (large circles) and the control value (dashed line), is more or less the same in (a) and (b) *i.e.* the measured number of dsb generated by *Pvu* II relative to the control values, was not affected by the plating procedure. This implies that neutral elution (pH 9.6) measures a background level of dsb due to the electroporation treatment only, upon which any further dsb are superimposed. This background level of dsb

was minimised by incubating the cells in tissue culture petri dishes and by selecting only those cells that remained attached during the ensuing incubation period for the neutral elution assay. In all subsequent experiments the treatment protocol was thus as follows: after electroporation, the samples were centrifuged, the medium aspirated and replaced with fresh medium, followed by incubation in tissue culture petri dishes.

In a final attempt to find a maxima in the level of dsb induced by *Pvu* II, incubation times of the assay were extended up to 24 h after electroporation and the results are shown in Fig 5.7.

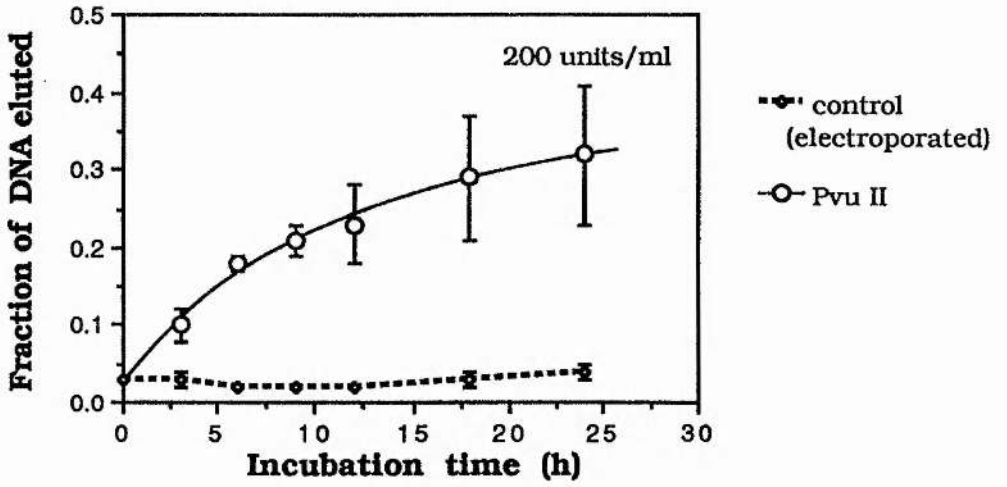


Figure 5.7 The induction of dsb in electroporated CHO cells treated with 200 units/ml *Pvu* II as measured by neutral elution (pH 9.6) up to 24 h post-treatment. These data represent the mean of 2 experiments and the vertical bars the standard error of mean values.

Despite the larger error values at longer incubation times it is evident that the numbers of dsb were still increasing over the 24 h post-treatment incubation period. The graph shows an initial relatively rapid increase in dsb up to 12 h, followed by a more gradual increase over 12-18 h and what appeared to be a levelling off of the curve between 18 and 24 h. The numbers of dsb due to electroporation treatment only (control

data), where only the attached cells had been selected for the neutral elution assay, were comparatively low and remained more or less constant over the 24 h time period.

The possibility that the increasing elution at the extended incubation times reflects DNA degradation rather than incision of the DNA by *Pvu* II, was tested using two assays on cells after incubation times of 7, 12 and 24 h. A trypan blue dye exclusion assay, where non-viable (permeable) cells show up blue, revealed less than 0.5 % cell 'death' in the population that remained attached to the petri dishes in both the electroporated controls and enzyme treated samples. Furthermore, the DNA of the electroporated controls and of the enzyme treated samples was found to be not soluble in TCA (small fragments, characteristic of DNA degradation would be soluble in 5 % TCA), which was further evidence against the possibility of DNA degradation (P. Bryant personal communication).

An experiment in which electroporated cells were treated with 200 units/ml *Pvu* II and incubated for up to 12 h, was repeated 5 times and the mean of the data is shown in Fig. 5.8, together with data (mean) obtained from 2 experiments in which electroporated CHO cells were treated with 200 units/ml *Bam* H1 and *Eco* R1.

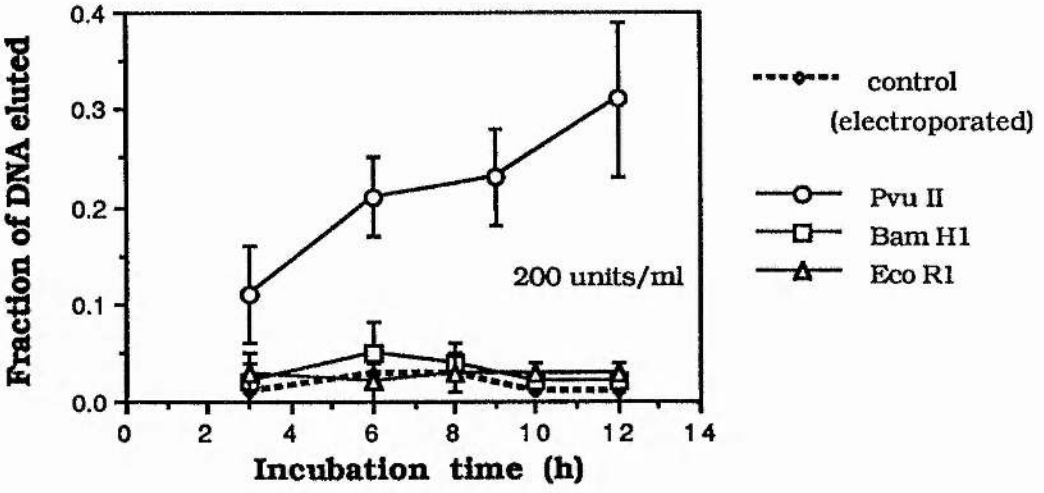


Figure 5.8 The levels of dsb in electroporated CHO cells treated with 200 units/ml *Pvu* II, *Bam* H1 and *Eco* R1 measured by neutral elution (pH 9.6) as a function of incubation time after treatment. The data represent the mean of at least two experiments and the vertical bars the standard error of mean values.

It is evident that for cells treated with *Pvu* II, a gradual increase in the number of dsb was measured over the 3-12 h incubation period in contrast to the cells treated with *Bam* H1 and *Eco* R1. Cells treated with the latter RE showed essentially constant and low levels of dsb which were not significantly different from that of the control (electroporated) samples.

In Fig. 5.9 the levels of dsb induced by *Pvu* II in the presence and absence of 400 µmol/l ara A were measured over a 3-12 h incubation period. The *Pvu* II data is the same as shown in Fig. 5.8. The data from the samples electroporated in the presence of *Pvu* II and 400 µmol/l ara A and incubated in the presence of 400 µmol/l ara A represent a single experiment but are supported by the results of other similar experiments.

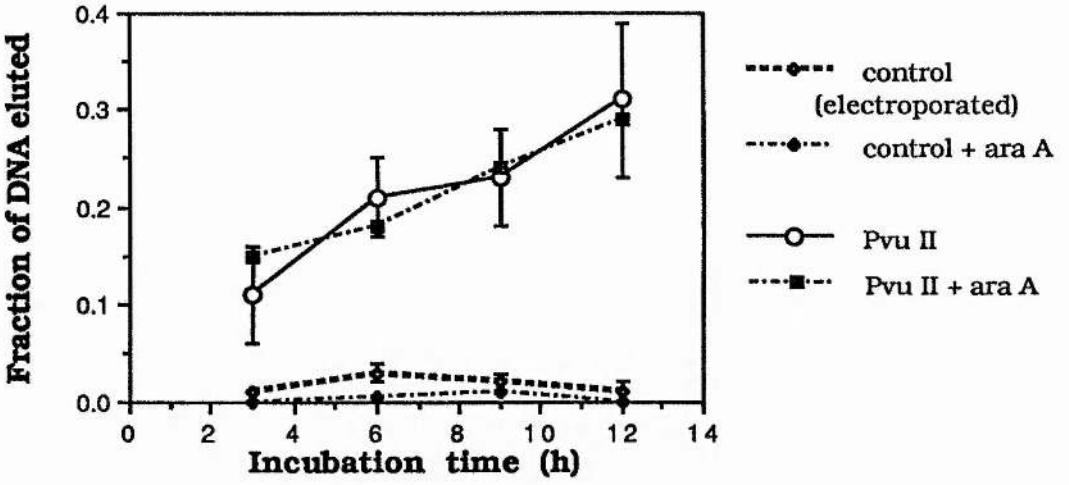


Figure 5.9 The levels of dsb as measured by neutral elution (pH 9.6) in electroporated CHO cells treated with 200 units/ml *Pvu* II in the presence and absence of 400 μmol/l ara A.

The presence of ara A, a known inhibitor of DNA synthesis, did not lead to a more rapid or higher accumulation of dsb in the *Pvu* II treated cells. The presence of 400 μmol/l ara A in the control samples gave consistently lower elution values than those obtained in the absence of the drug, but at these low levels of DNA damage it was hard to judge the significance of this observation.

5.4 Discussion

The observed increase in the fraction of DNA eluted in cells that had been permeablised to RE by electroporation, serves as verification that the non-denaturing filter elution assay (pH 9.6) undoubtedly detects dsb in mammalian DNA. The assay exhibited a linear increase in the fraction of DNA eluted, and thus in the level of dsb with *Pvu* II concentration (shown in Fig. 5.4) which supports the evidence of Winegar *et al.* (1989a) that electroporation is an effective method of introducing

RE into CHO cells. In Fig. 5.5 it can be seen that the elution profiles of the DNA from *Pvu* II treated cells and X-irradiated cells show very similar elution kinetics, which indicates that the mechanism of neutral elution is not dependent on the agent inducing the dsb. The neutral elution assay at pH 9.6 did however detect a significant amount of DNA damage due to the electroporation treatment alone, which was not observed in the cytogenetic assay used by Winegar *et al.* (1989a). The level of measurable dsb due to RE was therefore superimposed on this 'background' level of breaks (shown as control data throughout). In Fig. 5.6 it is shown how the background level of dsb was minimised by selecting only those cells that had remained attached to the tissue culture dishes during incubation for the dsb assay.

A maximum in the dsb induction curve by *Pvu* II was sought beyond which it might have been possible to measure dsb repair kinetics (for example after *Pvu* II had ceased to incise DNA), but as the post-treatment incubation times were extended from 1 up to 12 h then further up to 24 h, the numbers of dsb were found to continue increasing. Fig. 5.7 shows this increase to be relatively rapid up to 12 h, followed by what appeared to be a more gradual increase from 12-18 h and the 'induction' curve appeared to level off between 18 and 24 h.

No evidence of cell or DNA degradation was found in the electroporated and RE treated samples of attached cells over the 24 h incubation period, as tested by the ability of these cells to exclude trypan blue dye and by the solubility of DNA of the cells in 5 % TCA. It was therefore concluded that the continued increase in dsb indicated that the RE was actively incising DNA in the cell for at least 24 h following electroporation. An *in vitro* study in our laboratory has shown *Pvu* II to be stable at 37 °C for up to 24 h in HBSS (S. Moses personal communication). It is nevertheless surprising that the enzyme remains

active *in vivo* for up to 24 h. In this respect the action of RE differs significantly from that of a single acute dose of ionising radiation which largely induces prompt damage, although dsb may be generated later due to incision of double base damaged sites (Ahnström and Bryant 1982).

The shape of the *Pvu* II induction curve (Fig. 5.7) might be explained if it is assumed that repair of the dsb is occurring simultaneously with the incision of DNA by *Pvu* II, and that the *Pvu* II is cutting the DNA at a higher rate than that at which repair can take place. In other words, a competition could exist in the cell between enzymatic incision of DNA by Pvu II and repair of the induced dsb which is tipped in favour of incision at the enzyme concentration of 200 units/ml. As the incision rate by *Pvu* II slows down with time after electroporation treatment (perhaps due to gradual reduction in activity of the RE), the repair rate might gain on the incision rate and hence result in the levelling off of the induction curve.

The results in Fig. 5.9 show that the presence of 400 μmol/l ara A (a known inhibitor of DNA synthesis) did not affect the numbers of dsb induced by *Pvu* II, which in view of the above hypothesis of simultaneous incision and repair, would imply that the repair of these blunt-ended breaks does not require DNA polymerisation. Perhaps the blunt-ended termini of *Pvu* II-induced dsb require only simple ligation to be rejoined.

The most interesting result from this work was the comparison of levels of dsb induced by *Pvu* II and *Bam* H1 (see Figs. 5.3, 5.4 and 5.8). Assuming that *Bam* H1 is gaining access into the cells and therefore cutting the DNA as readily as *Pvu* II after the electroporation treatment, the lack of detection of the *Bam* H1 induced breaks might be due to the cohesive-ended nature of the termini. To ensure that this distinction between the action of RE which induce either blunt- or cohesive-ended dsb lay in the nature of the termini of the breaks, another RE which

generates cohesive-ended dsb, namely *Eco* R1, was also investigated. The outcome, as seen in Fig. 5.8, was clearly the same as was found for *Bam* H1 *i.e.* an inability of *Eco* R1 to generate measurable dsb.

The finding that *Bam* H1 and *Eco* R1 were less effective in inducing measurable dsb than *Pvu* II is in contradiction with the earlier results of Bryant (1984). Using the DNA unwinding assay, Bryant (1984) found that equal concentrations of *Pvu* II and *Bam* H1 induced strand breaks with similar frequencies. This discrepancy could be due to the fact that Bryant (1984) had measured the numbers of dsb at short incubation times after treatment with RE and since the enzymes had only limited time to act on the DNA the frequencies of breaks would have been low.

On the other hand, the neutral elution results are in agreement with cytogenetic studies which have shown RE that generate cohesive-ended breaks to be far less effective in giving rise to chromosomal aberrations than RE that produce blunt-ended breaks (Bryant 1984; Natarajan and Obe 1984; Winegar and Preston 1989a; Bryant and Christie 1989). It must be noted that the chromosomal aberration assay is a more sensitive technique and hence that lower concentrations of enzyme/1.10^6 cells were generally used.

Possible reasons for the lack of detection of the *Bam* H1 and *Eco* R1 induced breaks using the neutral elution assay (pH 9.6) are as follows:

1) The lower cutting frequency of *Bam* H1, of approximately once every 6000 bases, compared to every 3000 bases by *Pvu* II, could result in a lower number of dsb per unit activity of *Bam* H1. The *Eco* R1 data however dispels this idea as *Eco* R1 has a similar cutting frequency to *Pvu* II *viz.* about once every 3000 bases.

2) The four overlapping base-pairs, for both *Bam* H1 and *Eco* R1, prevent the full separation of the DNA fragments and therefore 'mask' the dsb during neutral elution. This is thought to be unlikely in the view of the

fact that the DNA experiences considerable shear forces during its passage through the 2 μm pores of the filter which would probably separate the hydrogen-bonded 4 base overlap (Hayward 1974)

3) The cohesive breaks are rejoined just as rapidly as they are induced by the RE. It is conceivable that if the DNA strands are held together by the 4 base overlap, the two breaks could rapidly be repaired as individual ssb rather than a dsb. In the case of an equilibrium between the incision rate and the repair rate, no dsb would be observed. This equilibrium would be dependent on the RE concentration, which might also explain the appearance of dsb at a *Bam* H1 concentration of 400 units/ml (Fig. 5.4) where presumably the repair rate falls behind the cutting rate at this very high concentration. This notion is further supported by the finding of Winegar *et al.* (1989b), in which CHO cells transfected with the *Eco* R1 gene could tolerate a certain level of constitutive expression of the gene (*i.e.* showed no increase in chromosomal aberrations), but additional insult (*e.g.* doses of ionising radiation) apparently over-burdened the repair system and enhanced cytogenetic damage was observed.

In conclusion, the hypothesis of a competition between RE incision and repair of the induced breaks would seem the most likely explanation for the results obtained in that it offers possible reasons for both the *Pvu* II and the *Bam* H1/*Eco* R1 data. This hypothesis can be tested by either treating the DNA with the RE in the lysate rather than in the cell, or by using a mutant cell line that is defective in dsb repair. The latter alternative was attempted and the results are presented in Chapter 6.

Finally, my neutral elution results of electroporated cells treated with RE would also suggest that blunt- and cohesive-ended dsb are repaired in different ways. The inability of a DNA synthesis inhibitor, ara A, to affect the repair of the dsb generated by *Pvu* II implies that these blunt-ended breaks might only require simple ligation to be rejoined.

Two possible models explaining the rejoining of cohesive-ended breaks have been suggested (Winegar and Preston 1988), namely direct ligation of the ends or excision of the cohesive overlaps followed by blunt ligation. The results presented here support the former model for the very reason that a difference in the level of *Pvu* II and *Bam* H1 induced breaks was observed. The results obtained in this chapter would seem to imply that cohesive-ended breaks are very rapidly repaired, and are possibly seen by the cell as two separate ssb.

CHAPTER 6

EXPERIMENTS USING THE XRS 5 MUTANT CELL LINE

6.1 Introduction

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6.1.2 Xrs mutants of the CHO K1 cell line

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6.2.3 Electroporation and RE treatment

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6.3 Results

6.3.1 Dose-response and repair results

6.3.2 RE treatment results

6.4 Discussion

6.1 Introduction

The xrs 5 mutant cell line was chosen since it is known to be deficient in dsb repair while normal in its ability to rejoin ssb as compared with the parental CHO K1 cell line (Kemp *et al.* 1984; Costa and Bryant 1988). It was the aim of this section of the study to investigate how the dsb repair deficiency of the xrs 5 cell line affected the detection of RE-induced dsb as measured by the non-denaturing filter elution assay (pH 9.6). These experiments were carried out in order to test the hypothesis, outlined in Chapter 5, of a possible competition between incision of the DNA by RE and dsb repair.

6.1.1 *Radiosensitive cell lines*

Radiosensitive cell lines have been used to shed light on the relationship between DNA damage and cell death after exposure to ionising radiation. Initial studies using a radiosensitive strain of the yeast *Saccharomyces cerevisiae*, the rad 52 strain, indicated that the increased sensitivity to ionising radiation was due to a deficiency in dsb repair (Ho 1975; Resnick and Martin 1976). This was followed by similar studies on mammalian cell lines, using either inherently radiosensitive cells *e.g.* from individuals with ataxia telangiectasia (Lehmann and Stevens 1977; Weibezahn and Coquerelle 1981; Cox 1982; Thierry *et al.* 1985) or mutants selected specifically for their radiosensitivity, such as the xrs and XR strains of the CHO cell line (Jeggo and Kemp 1983; Stamato *et al.* 1983), *irs* and V-series of mutants of the Chinese hamster V79 cell line (Jones *et al.* 1987; Zdzienicka *et al.* 1988; 1989) and the radiosensitive murine leukaemic lymphoblast line L5178Y-S (Alexander 1961; Nagasawa *et al.* 1980; Beer *et al.* 1983). The radiosensitivity of numerous mutant strains, for example of the xrs, L5178Y-S and *irs* lines, has been

attributed to a deficiency in dsb repair (Kemp *et al.* 1984; Wlodek and Hittelman 1988b; Zdzienicka *et al.* 1988; Costa and Bryant 1988; Whitmore *et al.* 1989). AT (ataxia telangiectasia) cells are however the exception in that they exhibit a similar dsb repair capability to that of normal human cell lines (Lehmann and Stevens 1977; Rahmsdorf *et al.* 1981; Thierry *et al.* 1985; Zdzienicka *et al.* 1989), and are thought to be radiosensitive as a result of low fidelity of repair of the dsb (*e.g.* Cox *et al.* 1986). Radiosensitive mutants are clearly useful in investigating the role played by dsb in chromosome damage and cell lethality.

6.1.2 *Xrs mutants of the CHO K1 cell line*

In 1983 Jeggo and Kemp reported the successful isolation of 6 appreciably X-ray-sensitive (xrs) mutant strains after mutagenic treatment of the CHO K1 cell line with ethyl methane sulphonate (EMS). They proceeded to show, using the neutral elution technique, that the increased radiosensitivity of these mutant lines was due to a deficiency in dsb rejoining (Kemp *et al.* 1984). The xrs mutants were also shown to exhibit enhanced chromosomal sensitivity to X-rays (Kemp and Jeggo 1986; Bryant *et al.* 1987; Darroudi and Natarajan 1987a; 1987b) and to RE induced dsb (Bryant *et al.* 1987), which correlates well with the dsb repair deficiency.

Genetic analysis revealed that all 6 mutant cell lines are genetically recessive and lie in single complementation group (Jeggo 1985). A very high reversion frequency was observed in the xrs strains after treatment with azacytidine (a powerful inhibitor of DNA methylation), which suggested that a xrs^+ gene was structurally intact but methylated and therefore not normally expressed (Jeggo and Holliday 1986). Jeggo and Holliday (1986) thus proposed that 2 copies of the xrs^+ gene exists in the parent CHO K1 line, but that due to methylation one copy is silent and

therefore that the CHO K1 line is functionally hemizygous for this gene. A mutation within the copy of the xrs^+ gene that is normally not methylated and therefore expressed in the wild-type CHO K1 line, is thought to be the cause of the repair defect in the xrs mutants. Following azacytidine treatment, which prevents methylation of replicated DNA, the silent and intact copy of the gene can subsequently be expressed (Jeggo and Holliday 1986) and hence reversion to the repair proficiency of the wild-type CHO cells can occur. The fact that all the xrs mutants lie within a single complementation group implies that all 6 xrs lines have a similar mutation.

The xrs 5 mutant cell line used in this study has been shown to be exceptionally radiosensitive (Jeggo and Kemp 1983; Costa and Bryant 1988) and of all the xrs strains it exhibits the most extensive deficiency in dsb repair (Kemp *et al.* 1984). The inability of the xrs 5 mutants to rejoin X-ray induced dsb has been determined using the non-denaturing filter elution technique (Kemp *et al.* 1984; Whitmore *et al.* 1989) and was verified by Costa and Bryant (1988) using the DNA unwinding method. The xrs 5 cells have also been found to show a 6-fold reduction in ability to carry out homologous recombination when compared to the parental CHO K1 cell line (Moore *et al.* 1986), although this was not found to be true for all the xrs strains (Hamilton and Thacker 1987).

6.2 Materials and methods

6.2.1 *Cell culture and labelling*

Asynchronous populations of exponentially growing xrs 5 mutant cells were routinely maintained in Eagle's minimal essential medium (MEM) supplemented with 10 % v/v calf serum (to which 100 μmol/l

$FeCl_3$ had been added). Essentially the xrs 5 cells were cultured in the same manner as the CHO parent cell line, except that the xrs 5 cells were seeded at double the number of CHO cells, *viz.* 2.10^6 cells (in 75 cm^2 plastic tissue culture flasks), since the doubling time of the xrs 5 line was found to be about twice that of the parent CHO K1 line. The amount of radioactive label however was not increased as the xrs 5 cells showed enhanced uptake of ^{3}H-TdR (Costa 1987) and hence the cells (2.10^6) were labelled with 3.7 KBq/ml ^{3}H-TdR (1.59 TBq/mmol) for 48 h.

6.2.2 *X-irradiation: dose-response and repair experiments*

After trypsinization the xrs 5 cells were X-irradiated in suspension in MEM (5.10^5 - 1.10^6 in 3 ml) at a dose-rate of 5.8 Gy/min.

For the dose-response experiments the samples were kept on ice for ~0.5 h before irradiation, irradiated on ice and returned to ice until further processing. For the short term (*i.e.* 0-3 h) repair experiments the samples were held at 37 °C in a water bath before irradiation, irradiated at 37 °C and returned to the water bath for the appropriate repair times. The irradiations were staggered such that all samples were collected at a common time point, at which time the cells were poured into ~10 ml ice-cold PBS and were kept on ice for further processing.

For the long term (*i.e.* up to 8 h) repair experiments, the cells were irradiated at 37 °C, then plated out into non-tissue culture petri dishes and placed in a humidified 37 °C, 5 % CO_2 incubator for the required repair times. Once again the irradiations were staggered such that the samples were collected at a common time point. At such time the cells were harvested and mixed with ~10 ml ice-cold PBS for the filter elution step.

The above procedure has been described in more detail in section 3.2.

6.2.3 *Electroporation and RE treatment*

The xrs 5 cells were electroporated and treated with purified restriction endonucleases (RE) in exactly the same manner as the CHO cells, which is described in detail in section 5.2.3. Briefly, 1 ml of cells at a concentration of 1.10^6/ml in MEM and to which the enzyme (200 units) had been added, were electroporated at 650 V/cm and 1600 μF (Winegar *et al.* 1989a). The samples were then centrifuged, the medium aspirated and the cells plated out in tissue culture petri dishes in fresh medium. The samples were then placed in a humidified 37 °C, 5 % CO_2 incubator for up to 12 h. The electroporation treatments were staggered such that all the samples were collected at a common time point, at which time the **attached** cells were harvested (by trypsinization) and mixed with ~ 10 ml ice-cold PBS for the neutral elution step.

6.2.4 *Non-denaturing filter elution assay (pH 9.6)*

The neutral elution procedure as described in section 2.2.2 was performed at pH 9.6 and under the lysis and eluting conditions stipulated in section 2.4.

6.3 Results

6.3.1 *Dose-response and repair results*

The spontaneous level of dsb in the control (unirradiated) samples was found to be consistently higher by approximately 5 % for the xrs 5 cells than for the CHO cells.

The induction of dsb in xrs 5 cells as a function of X-ray dose is shown in Fig. 6.1, and the CHO K1 data from Fig. 3.4 have been included

(denoted by the dashed line) to serve as comparison. The background elution values (unirradiated samples) have been subtracted in both cases.

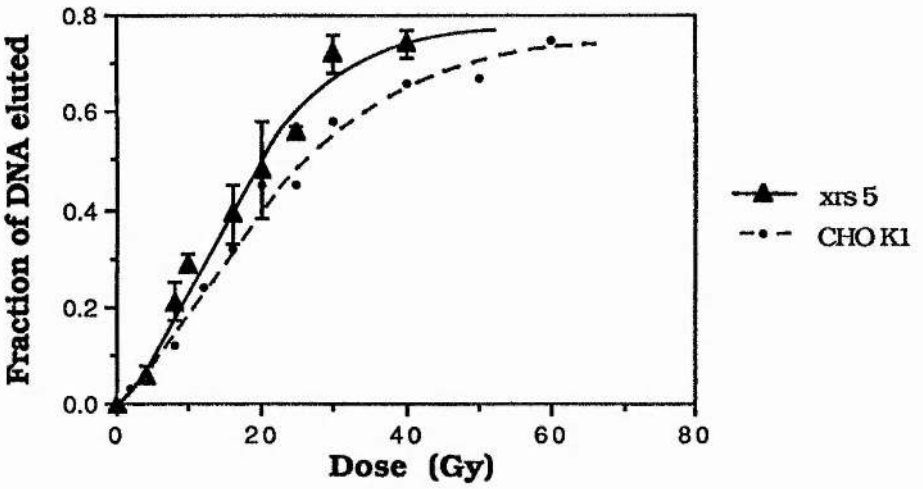

Figure 6.1 The dose-response for X-irradiated xrs 5 cells as measured by neutral elution (pH 9.6). The fraction of DNA eluted at 16 h was used and the points represent the mean of 3 experiments and the vertical bars the standard error of mean values. The CHO K1 data was taken from Fig. 3.4.

The xrs 5 mutants showed a marginally higher level dsb induction at each X-ray dose than the CHO cells, although it should be noted that the xrs 5 data points mostly lie just beyond the error bars of the CHO data points. There is a hint of the shoulder in the low dose region of the xrs 5 dose-response curve, but additional measurements would be necessary to substantiate this. As for the CHO curve, the xrs 5 dose-response tends to plateau out at high doses and this occurred at approximately the same level of elution but at a slightly lower dose value than in the CHO K1 dose-response curve (Fig. 6.1).

The repair kinetics of the xrs 5 cell line after a dose of 30 Gy as measured by neutral elution at pH 9.6 is shown in Fig. 6.2. In panel (a) the elution values have been plotted against incubation time after exposure and in (b) the data from (a) was plotted as % breaks rejoined

(see section 3.2.2). The CHO K1 data from Fig. 3.6 have been included to serve as comparison and are denoted by the dashed line.

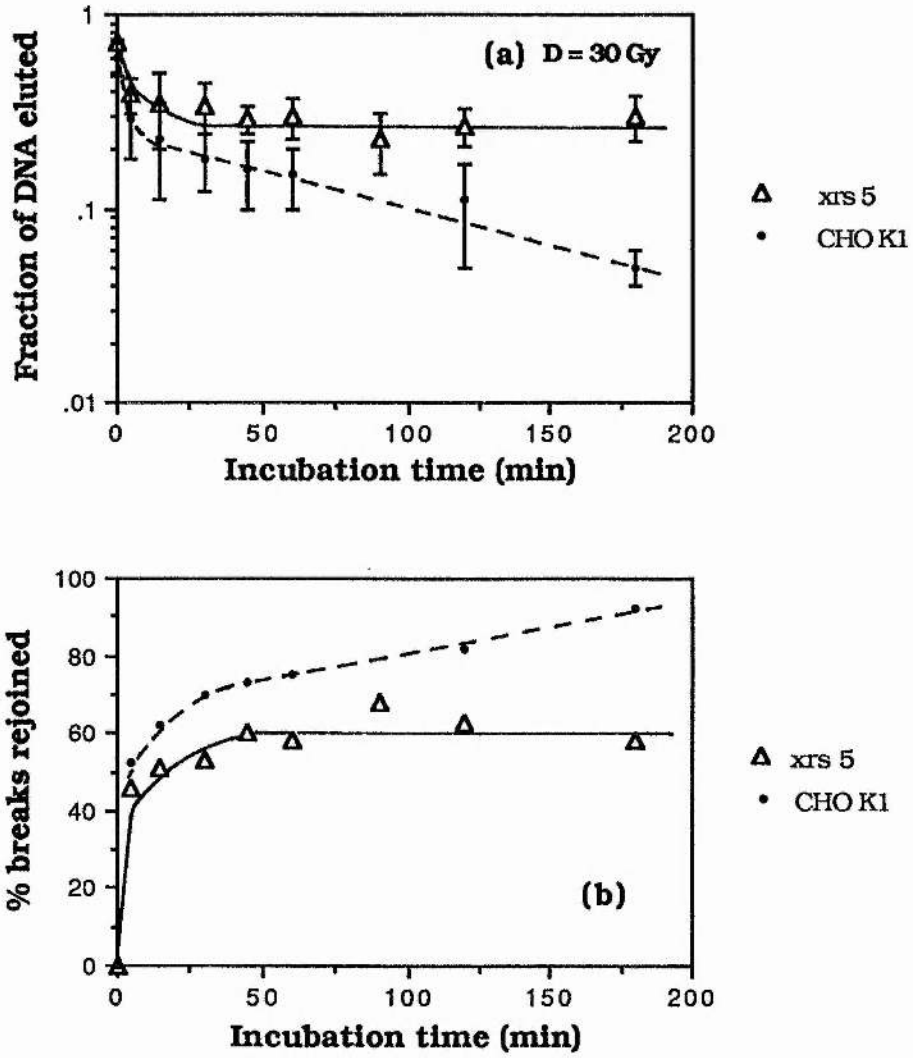

Figure 6.2 Kinetics of disappearance of DNA strand breaks in xrs 5 cells measured by neutral elution (pH 9.6) as a function of incubation time after X-ray exposure (30 Gy). The data points represent the mean of 5 experiments and the vertical bars the standard error of mean values. In (b) the data from (a) is calculated as % breaks rejoined. CHO K1 data was taken from Fig. 3.6.

The xrs 5 cells were found to be slightly less proficient in dsb repair within the first hour after exposure and show no evidence of repair (*i.e.* no decrease in numbers of dsb) between 1 and 3 hours post-irradiation, in contrast to a continuing decrease in the elution data of the CHO K1 cell

line over the 3 h incubation time. At 3 h post-exposure, ~ 60 % of the breaks had rejoined in the xrs 5 cells as compared with ~ 90 % repair in the CHO K1 line. The difference in the repair capabilities of the xrs 5 and CHO cell lines observed here was however not nearly as marked as had been reported by Kemp *et al.* (1984), even though the same dsb assay was used.

Repair experiments using xrs 5 and CHO K1 cells were performed in which the levels of dsb were followed up to 8 h after a dose of 75 Gy and the results are shown in Fig. 6.3.

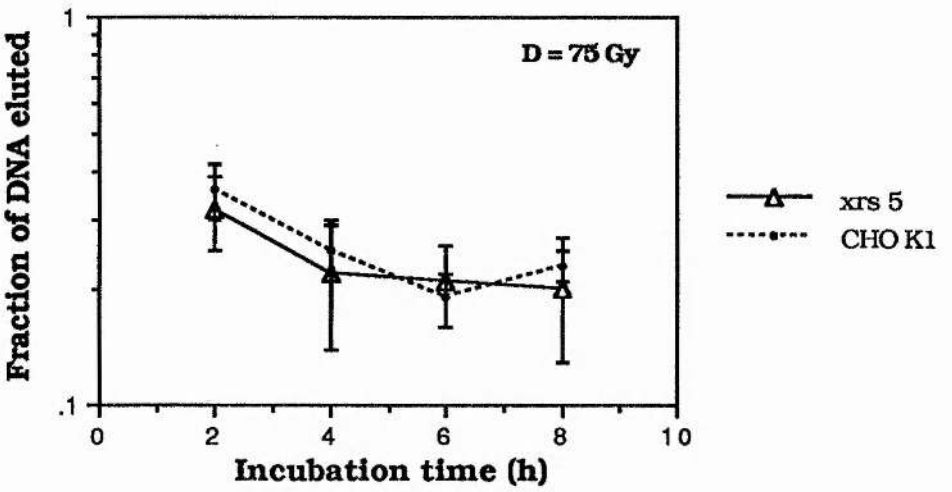

Figure 6.3 Kinetics of disappearance of DNA strand breaks measured in xrs 5 cells by neutral elution (pH 9.6) as a function of incubation time after X-ray exposure (75 Gy). The data points represent the mean of 2 experiments and the vertical bars the standard error of mean values.

Surprisingly the repair in the two cell lines were virtually indistinguishable, in contrast to the tendency shown in the former short term repair experiment (Fig. 6.2). It was then suspected that the xrs 5 mutants had in fact reverted to the wild-type level of radioresistance.

A batch of the original xrs 5 cell line, obtained from Dr P.A. Jeggo, was defrosted and cultured. The repair results obtained using these 'original' xrs 5 cells after a X-ray dose of 50 Gy is shown in Fig. 6.4. In (a)

the elution values have been plotted against incubation time after exposure and in (b) the data from (a) was plotted as % breaks rejoined (see section 3.2.2). The CHO K1 data from Fig. 3.8 (denoted by the dashed line) have been included to serve as comparison.

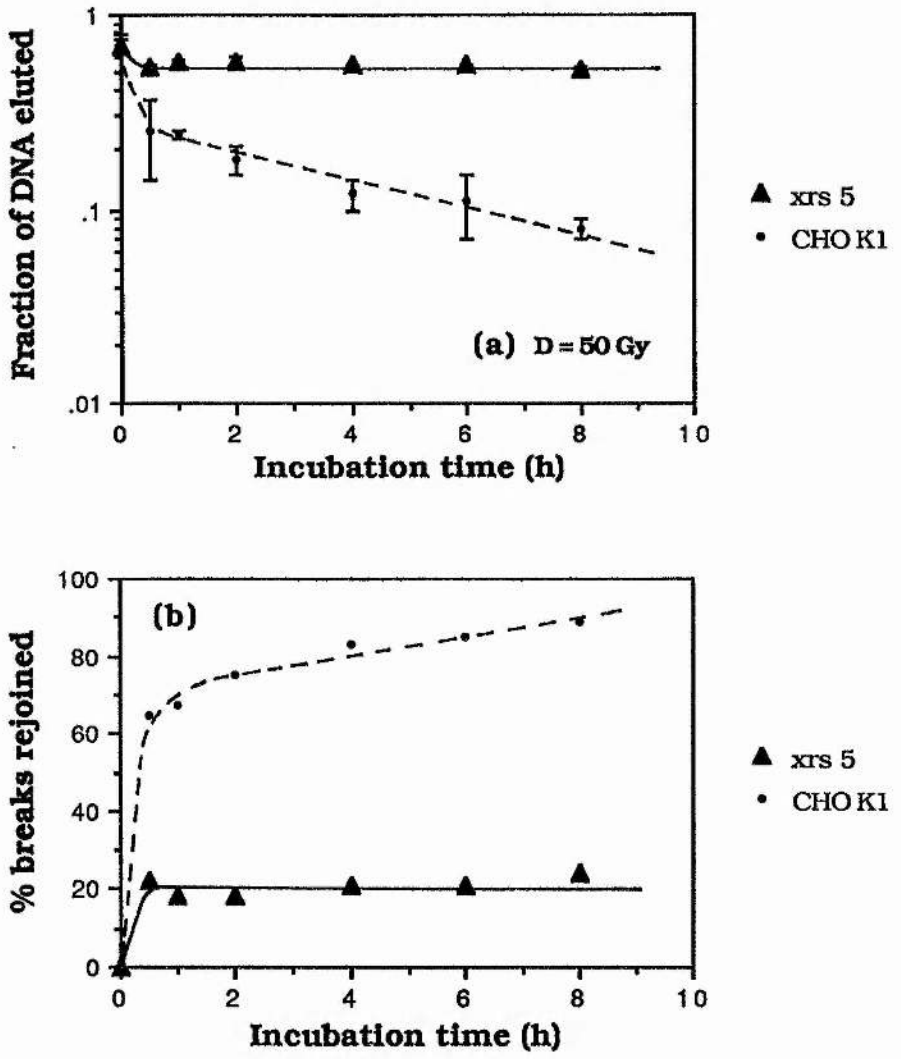

Figure 6.4 Kinetics of disappearance of DNA strand breaks measured in xrs 5 cells by neutral elution (pH 9.6) as a function of incubation time after X-ray exposure (50 Gy). The data points represent the mean of 2 experiments and the vertical bars the standard error of mean values. In (b) the data from (a) is calculated as % breaks rejoined. CHO K1 data was taken from Fig. 3.8.

Clearly there is a large difference in the repair capabilities of the wild-type CHO line and the 'original' xrs 5 mutants. From Fig. 6.4 (b) it can be

seen that 20 % of breaks had rejoined after 8 h in the xrs 5 cell line, in contrast to ~ 90 % in the CHO K1 cell line. It should also be noted that this limited amount of repair (20 %), which had taken place in the xrs 5 cells, had occurred within the first 30 min following exposure and that beyond this incubation time no further decrease in the levels of dsb was observed.

6.3.2 *RE treatment results*

The electroporation and RE treatment experiments were performed using the 'original' xrs 5 mutant cell line. Initial experiments in which the levels of RE-induced dsb were measured in electroporated xrs 5 cells, over short incubation times showed enhanced levels of dsb. The result of one such experiment is shown in Fig. 6.5.

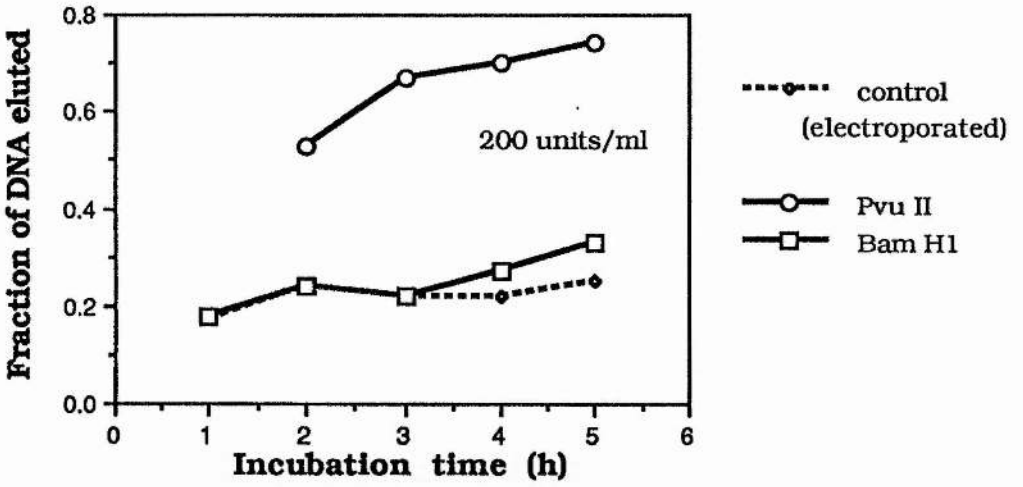

Figure 6.5 The levels of dsb in electroporated xrs 5 cells treated with 200 units/ml *Pvu* II or *Bam* H1 measured by neutral elution (pH 9.6) as a function of incubation time (in non-tissue culture dishes) after treatment.

The electroporation treatment alone (shown as control data; dashed line in Fig. 6.5) had a detrimental effect on a certain proportion of the xrs 5 cell population as was found for the parent CHO K1 line (Fig. 5.6). This

background level of breaks was minimised by selecting only the **attached** cells for the neutral elution assay (see Fig. 5.6 and section 5.4) and the result can be seen in Fig. 6.6. In these experiments, electroporated xrs 5 cells were treated with 200 units/ml *Pvu* II and *Bam* H1, and the kinetics of breaks were followed up to 12 h post-treatment using the neutral elution assay at pH 9.6. In panel (b) the CHO K1 data from Fig. 5.8 have been included to serve as comparison.

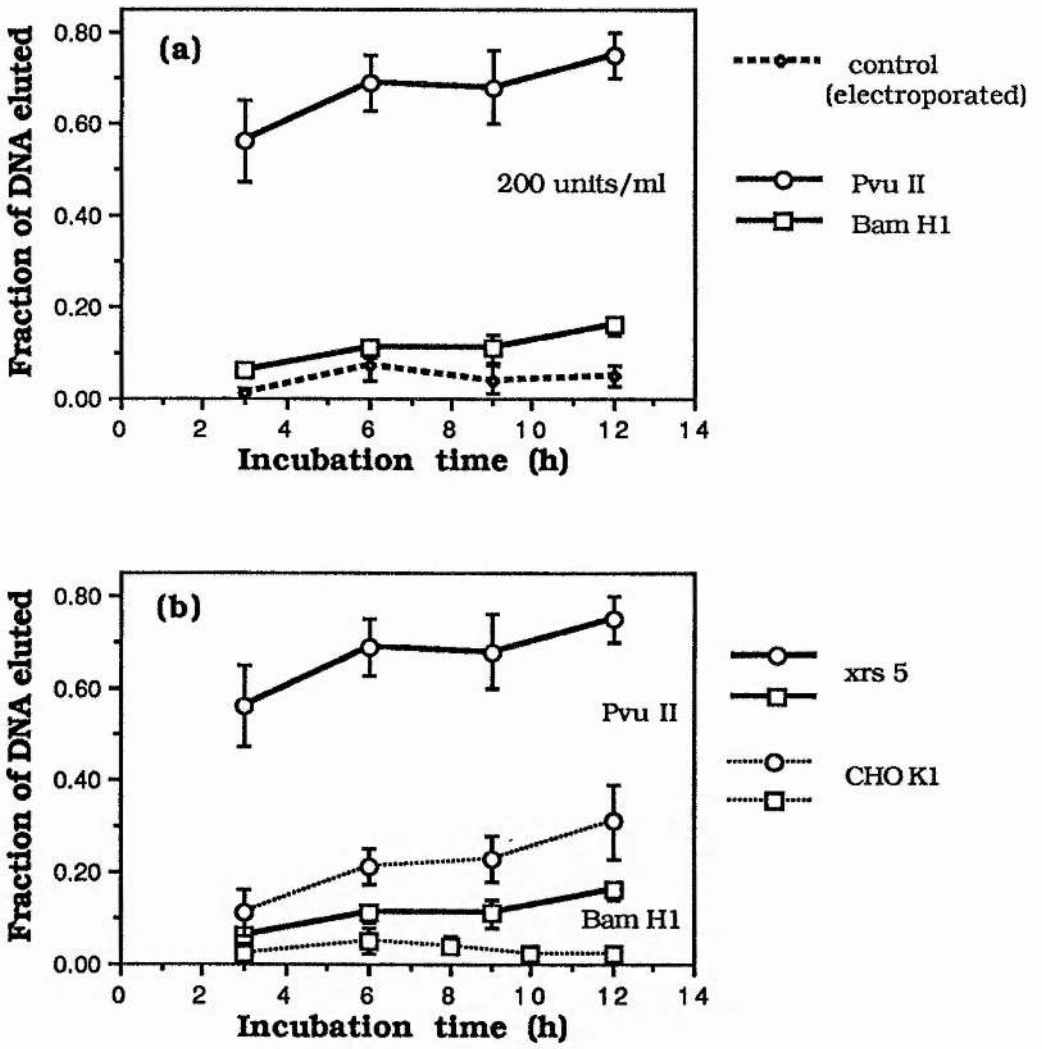

Figure 6.6 The levels of dsb in electroporated xrs 5 cells treated with 200 units/ml *Pvu* II or *Bam* H1 measured by neutral elution (pH 9.6) as a function of incubation time (in tissue culture dishes) after treatment. The data points represent the mean of 2 experiments and the vertical bars the standard error of mean values. In (b) the xrs 5 data from (a) is compared with CHO K1 data taken from Fig. 5.8.

The electroporated control samples of xrs 5 cells showed a low and more or less constant level of dsb over the incubation time that is almost identical to the CHO control data (see Fig. 5.7). From Fig. 6.6 (a), it is evident that *Pvu* II was considerably more effective than *Bam* H1 in inducing measurable numbers of dsb in the xrs 5 mutants (~3-4 times more effective). The *Bam* H1 treated xrs 5 cells showed levels of dsb that were significantly higher than measured in the electroporated control samples. In both the *Pvu* II and *Bam* H1 treated xrs 5 cells, the numbers of dsb were still increasing after 12 h post-treatment incubation, although the *Bam* H1 data showed less of an increase than that of *Pvu* II.

From Fig. 6.6(b) it can be seen that the overall level of dsb in the cells treated with 200 units/ml *Pvu* II were considerably higher in the xrs 5 mutant cell line than in the wild-type parent line. This is also true, but to a lesser extent, for the *Bam* H1 treated xrs 5 and CHO K1 cells.

6.4 Discussion

The induction of dsb by X-rays as measured by non-denaturing filter elution (pH 9.6) was found to be marginally higher in the xrs 5 cell line than in the parental CHO K1 line. On the one hand, this result is supported by other authors who have reportedly observed greater induction of dsb in radiosensitive strains (*e.g.* Wlodek and Hittelman 1987; Kelland *et al.* 1988; Tofilon *et al.* 1989), but it contradicts previous results obtained with the xrs 5 mutant (Costa and Bryant 1988; Iliakis *et al.* 1988). Costa and Bryant (1988) had found no difference in the level of dsb induced between the two cell lines with the DNA unwinding technique, while Iliakis *et al.* (1988) showed a similar result using the

neutral elution assay at pH 9.6. Iliakis *et al.* (1988) had in fact used the same technique as was employed in this project, albeit under somewhat different lysis and eluting conditions. It is hard to envisage a reason for greater induction of dsb in the xrs 5 cell line from a purely physical point of view (*i.e.* energy depositions in the cell), since one assumes the genome size in xrs 5 cells to be nearly identical to that the wild-type cells. Chromatin structure is also known to influence the extent of radiation damage induced (Chui *et al.* 1987; Warters *et al.* 1987; Heussen *et al.* 1987), but it is not clear why the chromatin structure should differ between the xrs 5 and CHO K1 cell lines. It is possible that the sulphydryl or glutathione content of the two cell lines are different, in which case the number of dsb resulting from X-ray exposure and measured by neutral elution would, according to Radford (1987b), not necessarily be the same

The marginal difference in the frequency of induced dsb measured in the xrs 5 and CHO K1 cells (shown in Fig. 6.1) is not nearly as marked as the difference between the respective radiosensitivities of the two cell lines. In this respect therefore, my data do not support Radford's hypothesis of a correlation between the level of dsb induction and cell killing (Radford 1985).

From the results shown in Figs. 6.2 and 6.3, where the difference in the extent of repair in the two cell lines was negligible or at least much smaller than would be expected from the data of Kemp *et al.* (1984), I was alerted to the fact that the xrs 5 mutants had possibly undergone reversion. Reversion of the xrs 5 cells, to a level of radioresistance similar to that of the wild-type CHO K1 cell line, had occurred previously in our laboratory after 6-9 months of continuous culture (Costa 1987). Although Jeggo and Kemp (1983) maintained that the isolated mutant lines were stable with regard to phenotype, it was later reported that the

xrs 6 strain had in fact reverted to a level of radioresistance similar to that of the wild-type cell line (Denekamp *et al.* 1989). The reversion of the xrs 5 mutant cell line could possibly be due to loss of methylation of the silent copy of the xrs$^+$ gene, which once expressed would allow the cells to reattain the level of dsb repair competence of the CHO cells.

A batch of the original xrs 5 cells kindly obtained from Dr P.A. Jeggo were taken out of liquid N_2 storage, defrosted and cultured. A rapid micro-nuclei assay, performed by a colleague (S. Moses), verified the reversion of the 'old' xrs 5 mutants and also found the freshly thawed xrs 5 cells to be extremely radiosensitive.

In the repair experiments performed with the 'original' xrs 5 cell line (see Fig. 6.4), a limited proportion of breaks (20 %) were rejoined within the first 0.5 h following irradiation, but no further decrease was monitored in the subsequent 0.5-8 h incubation period. Two possible explanations can be put forward for the initial extent of repair observed in the xrs 5 mutants; *viz.* (a) the total population of xrs 5 cells could have a limited capability of dsb rejoining, or (b) a limited proportion of xrs 5 cell population are fully capable of dsb repair. The latter notion is suggested by the model of Denekamp *et al.* (1989), in which transient expression of the xrs$^+$ gene is thought to occur in xrs cells when the silent copy of the gene is temporarily hemimethylated *e.g.* during or shortly after DNA replication. This model implies that a certain proportion of xrs 5 cells in the S-phase of the cell cycle would be temporarily proficient in dsb repair and therefore that at any one time, a fraction of exponentially growing xrs mutants will be capable of dsb repair. According to this hypothesis, the 20 % of repair observed might possibly reflect the proportion of xrs 5 cells that are in S-phase and which can therefore rejoin DNA dsb. This hypothesis does not however explain why no 'slow' repair (between 1 and 8 h) was detected in the xrs 5 strain (Fig. 6.4), since the theory infers

that a limited proportion of the cells are fully capable of repair and therefore that they should exhibit both components of repair.

In view of the fact that the xrs 5 mutants are proficient in ssb rejoining (Kemp *et al.* 1984; Costa and Bryant 1988), the repair results shown in Fig. 6.4 thus provide evidence that the non-denaturing filter elution assay is not affected strongly by the presence of ssb; *i.e.* if the fast component of repair, as measured by neutral elution, reflected ssb rejoining it would be expected that the xrs 5 mutants might exhibit extensive 'fast' repair, which can be seen not to be the case from Fig. 6.4.

Results obtained by Thacker and Stretch (1985), after irradiating the xrs mutant lines with either γ-rays or α-particles suggests that the mutants are deficient mainly in the repair of damage from sparsely ionising radiation, which might suggest that the xrs 5 mutants are deficient in a specific pathway of dsb repair. Although the mechanisms of dsb repair are largely unknown, Moore *et al.* (1986) has found this mutant line to show a marked reduction in recombination frequency when compared with the parental CHO K1 cell line.

It is clear that the numbers of breaks measured in electroporated xrs 5 cells treated with 200 units/ml *Pvu* II are 3-4 fold higher than in the CHO K1 cells (Fig. 6.6). Also, 200 units/ml of *Bam* H1 gave rise to a number of dsb which was measurable above the control level in the xrs 5 mutants, unlike the *Bam* H1 results of the CHO K1 cells. Bryant *et al.* (1987) had found a similar tendency in a cytogenetic investigation of xrs 5 mutants treated with RE, where *Pvu* II caused considerably higher frequencies of chromosomal aberrations (CA) in the xrs 5 cells than in CHO cells and unlike CHO cells, xrs 5 mutants treated with *Bam* H1 exhibited higher than background levels of CA.

Two possible reasons can be put forward to explain the enhanced levels of RE-induced dsb measured in the xrs 5 mutant cell line:

1) It could be due to an increased cutting frequency of the RE in the xrs 5 mutants. This explanation is however unlikely if one considers the evidence that the xrs 5 mutant cell line is the result of a single mutation within the xrs$^+$ repair gene (Jeggo 1985; Jeggo and Holliday 1986) and that the genome and chromatin structure is otherwise thought to be identical to that of the parental CHO K1 line. Thus the numbers of RE sites and therefore the cutting frequency should be essentially identical in the two cell lines.

2) Assuming the model of a competition between RE incision and repair of the resulting dsb (as suggested in section 5.4), the diminished dsb repair capacity of the xrs 5 mutants would tip the balance more strongly in favour of incision which would result in enhanced levels of dsb.

The results obtained from the electroporation and RE experiments performed with the xrs 5 mutant line which show 3-4 fold higher levels of measurable dsb than in the CHO cell line, thus strongly supports the competition hypothesis. According to this model, the results shown in Fig. 6.6 suggest that the xrs 5 cell line is deficient in the ligation repair required for the rejoining of the blunt-ended dsb induced by *Pvu* II. The enhanced level of *Bam* H1-induced breaks observed in the xrs 5 mutants would suggest that a proportion of the cohesive-ended dsb may be repaired by a similar pathway to that of blunt-ended dsb. It is possible that the single-stranded ends of <u>some</u> cohesive-ended dsb are degraded and thereby converted to breaks with blunt-ends in the xrs 5 cell line (Smith-Raven and Jeggo 1989), although this did not appear to occur in the wild-type CHO K1 cell line (see Fig. 5.8).

CHAPTER 7

GENERAL DISCUSSION

7.1 Non-linear dsb induction results

7.2 A hypothesis based on DNA fragment size

7.3 Dsb repair kinetic results

7.4 Conclusions

The aim of this project was to attempt to test the proposed specificity of the non-denaturing filter elution technique as an assay for dsb in mammalian cells. After initial optimization of the lysis and eluting conditions, detailed dose-response and DNA repair experiments were carried out at pH 9.6 and 7.4 with the CHO K1 and xrs 5 cell lines. The effect of the DNA synthesis inhibitors, ara A and ara C, on putative dsb repair measured by neutral elution was investigated and the limited inhibition of repair that was measured is discussed in view of earlier reports of complete inhibition of dsb repair by these drugs as measured by neutral velocity sedimentation.

By treating electroporated cells with RE, it was demonstrated that the non-denaturing filter elution assay undoubtedly detects dsb. Time course studies on RE treated cells revealed that the enzyme remained active in the cell for a considerable length of time. A surprising outcome was the relative inability of the neutral elution assay to detect cohesive-ended dsb induced by *Bam* H1 or *Eco* R1, as compared to significant levels of blunt-ended dsb induced by *Pvu* II. A competition between RE incision and dsb repair is offered as explanation, and this is supported by the enhanced level of breaks monitored in the dsb repair deficient mutant cell line, xrs 5.

In this last chapter I would like to discuss these results in the context of the current hypotheses regarding the interpretation of data obtained with the non-denaturing filter elution assay, and to compare the results with those of the neutral velocity sedimentation technique. Possible interpretations of my results are put forward and discussed.

7.1 Non-linear dsb induction results

Using the non-denaturing filter elution technique as an assay for dsb in cultured mammalian cells following X-ray exposure, I obtained sigmoidal dose-response relationships for the CHO, xrs 5 and EAT cell lines (Figs. 3.4, 4.6 and 6.1). A concave shoulder was observed in the low dose region (0-5 Gy) while at high doses (40-60 Gy) the induction curves exhibited a tendency to plateau out. This shape of the dose-response curve was found to be independent of the pH of the assay (see Fig. 3.5).

The dose-response relationship of the non-denaturing filter elution assay is thought to represent the induction of dsb, and using this technique Blazek *et al.* (1989) demonstrated a parabolic increase in numbers of induced dsb with γ-ray exposure in V79 hamster cells. In the light of this result they suggested that dsb induction might be entirely a two-hit event. The sigmoidal shape of the dsb induction curves obtained during the course of this project does not appear to support the hypothesis of Blazek *et al.* (1989), since a distinctly linear increase in dsb was observed in the dose range between 8 and 30 Gy in all three cell lines (see Figs. 3.4, 4.6 and 6.1).

According to Okayasu and Iliakis (1989) the shoulder in the dose-response curve obtained with the neutral elution assay could be eliminated by employing more rigorous lysis conditions (*e.g.* by increasing the lysis temperature to 60 °C). On the basis of these results, they attributed the shoulder to incomplete separation of the DNA from the chromatin which would influence the elution kinetics at low doses of radiation, *i.e.* multiple hits might be required before the DNA pieces can be separated from the residual matrix structure. My results (Fig. 2.5) show that the use of the detergent NLS in conjunction with the elevated lysis temperature of 60 °C (as per Okayasu and Iliakis 1989), did lead to a

marked increase in the extent of DNA eluted from the filter (see Fig. 2.5), which would imply more effective separation of the DNA molecules from the associated proteins. Warters *et al.* (1987) and Wlodek and Hittelman (1987) had found that less than 2% of chromatin proteins could be detected on the filter after the lysis conditions of Bradley and Kohn (1979) and therefore one would expect considerably less to remain following the stronger lysis treatment of Okayasu and Iliakis (1989). It would therefore seem unlikely that the shoulder, in the dose-response curves presented in this thesis, is fully accounted for by possible residual chromatin structure.

In addition to the shoulder observed at low doses, a tendency of the dose-response curve to plateau out at higher doses was also observed (*e.g.* Fig 3.4). 10-15 % of DNA would not elute from the filter even at high doses and this proportion of non-eluting DNA was similar at both pH 9.6 and 7.4, and was observed in all three cell lines *i.e.* CHO, EAT and xrs 5 (see Figs. 3.5, 4.6 and 6.1). I suggest that this effect could be due to adsorption of the DNA on to the filter. Since cells in exponential growth were used in all of my experiments, it is also possible that this percentage of the DNA could form part of the complex system of forked structures associated with DNA replication that would be unaffected by the lysis conditions, as manifested by the reduced elution of DNA of S-phase cells (Okayasu *et al.* 1988).

Radford's interpretation of the shouldered dose-response relationship was that it mirrored the shouldered cell survival curves (Radford 1985; 1986). This would suggest that there is a direct relationship between the initial induced level of dsb and cell killing. My dose-response data obtained with the xrs 5 mutant cell line (Fig. 6.1) would refute this interpretation on two counts: Firstly the dsb induction curve of xrs 5 cells possibly showed a small shoulder, unlike the

exponential survival curve for these mutant cells (Jeggo and Kemp 1983; Costa and Bryant 1988). Secondly, although a marginal difference in the frequency of induced dsb was observed between the xrs 5 and parental CHO K1 cell lines, the difference does not correlate with the markedly different radiosensitivities of the respective cell lines (Jeggo and Kemp 1983; Costa and Bryant 1988). Radford (1987b) attributed the shoulder in the dsb induction curves to a saturable process of sulphydryl 'shielding' of the DNA from hydroxyl radicals, but this hypothesis is undermined by the fact that the size of the shoulder can be reduced or, as Okayasu and Iliakis (1989) reported, eliminated by simply altering the lysis treatment.

The data for induction of dsb as measured by non-denaturing filter elution, collected during the course of this project, does thus not support any of the above mentioned interpretations of the non-linear dose-response relationship. An alternative explanation for the shoulder at low doses is therefore offered which is based on DNA fragment sizes.

7.2 A hypothesis based on DNA fragment size

A shouldered dose-response following X-ray exposure of mammalian cells had also been observed by Blöcher (1990) using the CHEF electrophoresis technique and in the report he postulated that the non-linear induction of dsb could be explained in terms of DNA fragment sizes. His model is based on the assumption that the size of the untreated DNA strands (based on the average length of a chromosome of 245 Mbp) is considerably larger than the threshold size for movement out of the well. Consequently, it may require more than one dsb to reduce the fragment lengths to below the threshold size, and this would result in a shoulder in the low dose region of the dose-response curve.

Despite the obvious differences in the mechanisms of the CHEF electrophoresis and the neutral elution techniques, there are to my mind similarities which would make Blöcher's idea similarly applicable to the neutral elution assay. Neither of the techniques can resolve the sizes of the various DNA fragments (unlike the neutral velocity sedimentation assay), but are rather based on a 'sieving' effect of the DNA *i.e.* DNA fragments below a certain threshold size will move out of the well or be eluted whereas those of a size above this will be retained in the well or on the filter, and in both cases the results are presented as a fraction of DNA 'extracted' or eluted relative to the amount remaining in the well or on the filter. Blöcher's hypothesis could thus similarly be applied to the neutral elution assay on the assumption that the chromosome length of unirradiated DNA (~5 cm), as a free molecule, is considerably longer than the fragment size able to elute through the 2 μm pores in the filter. 5 cm is an estimate of the <u>average</u> chromosome length, but the fragment sizes of unirradiated DNA are likely to be some what smaller as a result of shearing of the DNA during handling or interruptions that occur in the strands at points of replication. It is suggested that multiple dsb (*i.e.* more than one) are required within a chromosome length of DNA to effect elution, thus giving rise to the shouldered dose-response of the neutral elution assay. Once the bulk of the DNA fragment sizes are below this threshold, the probability of elution would increase with a decrease in fragment length and therefore the shoulder is followed by a linear increase in the fraction of DNA eluted with dose. The DNA fragment lengths in questions would still be considerably larger than the micrometer dimensions of the diameter of the pores in the filter and thus the elution kinetics would not be expected to alter with varying pore size as was reported by Kohn (1986).

Clearly the above reasoning should also apply to dsb induction following exposure to high LET irradiation or RE treatment. The neutron data of Van der Schans *et al.* (1983) is insufficient to verify this, but closer inspection of the results of Prise *et al.* (1987), Prise *et al.* (1989a) and Fox and McNally (1988) would seem to suggest that shouldered dose-response curves are also obtained after exposure to neutrons and α-particles. On the other hand the linear induction of dsb by *Pvu* II in electroporated cells or by decays of incorporated ^{125}I (Radford and Hodgson 1985; Peak *et al.* 1988a) would seem to undermine this theory. The possibility that a shoulder in the *Pvu* II dsb induction curve is obscured by the background level of breaks due to electroporation cannot be excluded (see Fig. 5.4). In the case of ^{125}I-induced dsb the only reason that I can offer for the lack of a shoulder in the dose-response curves, is the fact that only limited data is available for low numbers of accumulated breaks (see Peak *et al.* 1988a). Closer inspection of Radford's (1987b) 'nuclear monolayer' dsb induction data also reveals the dose-response curves to be near-linear rather than linear *i.e.* exhibiting a reduced shoulder rather than none at all.

The neutral velocity sedimentation technique has been shown to resolve the size of DNA fragments, but Blöcher (1982) reported the requirement of a additional dose of 10 Gy of X-rays to the cells just prior to lysis to enable the determination of small numbers of dsb. This 'resolving' dose could conceivably obscure the possible detection of a shoulder in the low dose region with the neutral velocity sedimentation assay.

7.3 Dsb repair kinetic results

The dsb repair kinetic results obtained with the non-denaturing filter elution technique and presented in this thesis, are discussed below in the light of current hypotheses explaining the observed biphasic kinetics of repair. The dsb repair kinetic results of Blöcher and Pohlit (1982) as measured by neutral velocity sedimentation are borne in mind throughout. The possible interpretations of my results of monitoring the RE-induced dsb in electroporated cells are also discussed in terms of repair kinetics.

In contrast to the apparent first-order kinetics of dsb repair following exposure to ionising radiation with a half-time of some 2-4 h as measured by neutral velocity sedimentation (Blöcher and Pohlit 1982), the neutral elution assay detects biphasic repair kinetics, where 50 % of the breaks had disappeared within 30-40 min of exposure (Bradley and Kohn 1979; Kemp *et al.* 1984). Some investigators suggested that the biphasic kinetics reflect two distinct mechanisms of repair, which were for obvious reasons referred to as the 'fast' and 'slow' repair components (Weibezahn and Coquerelle 1981; Woods 1981). Estimates from my data of the half-times of these components are ~5 min and 1-3 h respectively (see Figs. 3.6-3.8).

In contrast to the full inhibition of dsb repair by the DNA synthesis inhibitors ara A and ara C, as demonstrated by Bryant and Blöcher (1982) and Iliakis and Bryant (1983) using the neutral velocity sedimentation and DNA unwinding techniques, the neutral elution assay showed only limited inhibition of repair in the presence of these nucleoside analogues. The fact that 80 % of rejoining had taken place in the presence of 400 µmol/l ara A or ara C (see Figs. 4.7 and 4.8), indicates that the repair mechanism of DNA dsb, as detected by neutral elution, does not have a absolute

requirement for DNA polymerization. These results would therefore suggest that the neutral velocity sedimentation and neutral elution techniques are detecting two disparate DNA dsb repair processes, *i.e.* neutral elution detects a type of ligation process which does not have a requirement for DNA polymerization while neutral velocity sedimentation detects a polymerase dependant repair pathway (Bryant and Blöcher 1982).

As mentioned previously, Weibezahn and Coquerelle (1981) and Woods (1981) proposed that the biphasic kinetics represented two distinct mechanisms of dsb repair, namely a fast ligation process and a slower repair mechanism, which possibly involves recombination. On the one hand therefore my ara A and ara C data supports the above hypothesis of the existence of more than one dsb repair mechanism, but with the difference that the two repair processes are inferred by the results of two different techniques and not necessarily by the two repair components of the neutral elution assay. Weibezahn and Coquerelle (1981) postulated that the fast component of repair (as assayed by neutral elution) represented a simple ligation process, whereas the slow component repair represented a more complex repair mechanism which possibly involved recombination between the homologous chromosomal regions. Results of the neutral elution study using DNA synthesis inhibitors presented here however indicate that neither the fast or slow components of repair showed significant inhibition (Figs. 4.7 and 4.8), which would suggest that even the slow repair component does not require DNA polymerization and thus also reflects a ligation process.

Alternatively, the fact that neither of the two repair components were preferentially inhibited could be interpreted to suggest that the biphasic kinetics are artefactual and somehow simply represent a single repair mechanism. The fast and slow components could for example

result from a difference in accessibility to different regions of the DNA within the chromatin structure, as suggested by Radford (1987a), *i.e.* repair enzymes could gain access to the less condensed euchromatin more readily than to the more compact heterochromatin. This hypothesis however is undermined by the first-order repair kinetics detected by neutral velocity sedimentation, *i.e.* if the notion of differential accessibility were true it should also be reflected in the neutral velocity sedimentation study of dsb repair kinetics.

Ahnström (Comment on Radford 1985) and Hutchinson (1989) have proposed that the fast component of repair represents ssb rejoining since the repair rate constants of the two are similar. This hypothesis is based on the assumption that the presence of ssb might increase the rate of elution of the double-stranded DNA, by increasing the flexibility of the DNA or by making the DNA fragments more susceptible to shearing. The ability of xrs 5 cells to rejoin ssb has been shown to be similar to that of the wild-type CHO cells (Kemp *et al.* 1984; Costa and Bryant 1988), and therefore the level of repair measured in the xrs 5 mutants provides strong evidence that neutral elution is not affected strongly by the presence of ssb. Otherwise it is hard to explain why the cell lines which do not differ in ssb rejoining ability, exhibit markedly different levels in the fast rejoining process (see Fig. 6.4). The notion that the fast repair component might reflect ssb rejoining is also counteracted by the hydrogen peroxide results, where large numbers of ssb, that were induced by H_2O_2, did not lead to measurable levels of elution of the DNA under non-denaturing conditions (Bradley and Kohn 1979; Prise *et al.* 1989b).

The above hypothesis might address the question of the biphasic versus the first-order dsb repair kinetics, but it does not clarify why the repair half-times as monitored by the neutral velocity sedimentation and

neutral elution techniques are so vastly different ($t_{1/2}$ = 2-4 h as compared to 30-40 min respectively). On the other hand, if the biphasic repair kinetics are assumed to reflect two distinct repair mechanisms, the half-time of the slow component of 1-3 h (see Figs. 3.6-3.8) is not unlike that of velocity sedimentation. This might suggest that the two techniques are detecting a common repair mechanism, but the results obtained with the DNA synthesis inhibitors refute this idea. This controversy is further complicated by the recent findings of Frankenberg-Schwager *et al.* (1990) in which biphasic repair was demonstrated in yeast after low doses of X-rays using the neutral velocity sedimentation technique. However, the validity of extrapolating this result to dsb repair in mammalian cells is questionable, especially in view of the fact that Blöcher and Pohlit (1982) had obtained first-order repair kinetics for doses from 40 Gy down to as low as 10 Gy, doses well within the range used here for the neutral elution assay.

A direct measurement of the rejoining of the RE induced dsb was not possible since the repair was obscured by what is postulated to be the continuing incision of the DNA by the enzymes over a 24 h post-treatment period. Details of the repair processes could however be inferred indirectly by the comparative study of RE which generate dsb with different types of termini.

The following deductions are based on the assumption that the observed kinetics of RE induced dsb (see Fig. 5.7) result from a competition between RE incision and dsb rejoining, an idea which appears to be justified by the enhanced levels of breaks detected in RE treated xrs 5 cells as compared with CHO cells (see Fig. 6.6). The five to tenfold difference in the levels of measurable dsb induced in CHO cells by *Pvu* II and *Bam* H1/*Eco* R1 respectively (see Fig. 5.7), would seem to indicate that the repair of cohesive-ended dsb (induced by *Bam* H1/ *Eco*

R1) takes place at a higher rate than blunt-ended dsb rejoining. It is not unreasonable to assume that *Pvu* II induced breaks would require a blunt-end ligation mechanism to be repaired (as inferred from the ara A experiments - see Fig. 5.9), while cohesive-ended breaks could be rejoined by a 'ssb-like' ligation repair process. It is plausible that the cohesive breaks could be regarded as two ssb in close proximity (should the two strands of DNA not separate) and could be acted upon as such by a DNA ligase. On the other hand, the data obtained with *Bam* H1 treated xrs 5 cells suggests that this is in fact an oversimplification of the repair mechanism of cohesive-ended breaks, since the xrs 5 cells exhibited a significant increase in measurable *Bam* H1 breaks, above those observed in the CHO K1 cell line, even though the xrs 5 cell line is fully proficient in ssb repair. The data obtained with these RE nevertheless suggests that the cohesive breaks are rapidly repaired *i.e.* by means of a process that is faster than blunt-end ligation, perhaps with similar kinetics to ssb repair, but which may also have some requirement for the repair machinery of dsb. Thus the investigation of dsb induced by RE in electroporated cells, as assayed by non-denaturing filter elution, has suggested on the basis of their respective repair rates that cohesive- and blunt-ended dsb might be rejoined by different repair mechanisms.

In the light of all the results obtained during the course of this project, it is proposed that the non-denaturing filter elution technique detects dsb which require simple repair processes *e.g.* ligation, while velocity sedimentation possibly detects dsb with dirty ends which require exonuclease action and subsequent DNA polymerization to be rejoined or possibly LMDS which require recombination (Ward 1985). It is difficult to envisage the reason why the two techniques should detect disparate types of dsb. Other than the differences in the biophysical mechanism of separation of the DNA fragments, the only major difference in the

experimental protocols followed, is the duration of the lysis treatment; which is 1 h in the case of the neutral elution assay compared with the 17-18 h lysis treatment of velocity sedimentation (Blöcher 1982). A highly speculative guess would be that the extended lysis treatment of velocity sedimentation could allow renaturation of 'clean' cohesive- or blunt-ended dsb and consequently these types of breaks are not observed. It is also difficult to explain why the neutral elution assay does not detect the repair mechanism (ara A sensitive) which is associated with DNA polymerization, unless a further third component of repair might be revealed in studies over longer (>5 h) incubation times with the filter elution technique.

7.4 Conclusions

The results obtained during the course of this project indicate that the non-denaturing filter elution technique unquestionably detects DNA dsb, but that the method possibly detects one or more dsb lesion(s) different from that assayed by neutral velocity sedimentation.

The sigmoidal dose-response curves obtained, considered in conjunction with the only marginal difference in the induction of DNA dsb in the CHO K1 and xrs 5 cell lines, would seem to indicate that the non-linear induction curves are probably an artefact of the assay rather than a reflection of a specific underlying mechanism of dsb induction.

The series of experiments in which DNA synthesis inhibitors (ara A and ara C) and *Pvu* II were used, point to simple ligation as the most likely mechanism of dsb repair that is detected by the neutral elution assay. On the basis of these results and other data obtained with *Bam* H1 and *Eco* R1, the following interpretations of the biphasic repair kinetics

of the non-denaturing filter elution assay following X-ray exposure are suggested:

(1) The two repair components could reflect the repair mechanisms of different types of dsb induced by sparsely ionising radiation. For example, in the event of the possible induction of cohesive-ended and blunt-ended dsb by X-rays, the initial repair component ($t_{1/2}$~5 min) of neutral elution could reflect the fast repair of cohesive-ended dsb, whereas the slow repair component of neutral elution ($t_{1/2}$ = 1-3 h) could represent the ligation of blunt-ended dsb.

(2) An alternative explanation could be that the biphasic repair kinetics are an artefact of the neutral elution technique and that the data reflects a single repair mechanism with a $t_{1/2}$ of 30-40 min. The fact that the CHEF electrophoresis also detects biphasic repair kinetics with a similar $t_{1/2}$ value implies that it could be an experimental artefact which is common to both these techniques, although the nature of this artefact is not clear.

As mentioned previously, the neutral velocity sedimentation technique might not detect the 'faster components' of repair if renaturation of these 'clean' (cohesive and blunt-ended) dsb could possibly take place during the extended lysis treatment employed. It is worth mentioning that the $t_{1/2}$ of the slow component of non-denaturing filter elution and that of neutral velocity sedimentation are not dissimilar, although my data (obtained with DNA synthesis inhibitors) would suggest that the mechanisms of repair that are measured by the two techniques are disparate. The hypothesis that the biphasic repair is due to differential accessibility of the repair enzymes to the DNA, as explained above, is refuted by the first-order kinetics obtained with neutral velocity sedimentation.

Finally it can be said that this study has provided strong evidence that ionising radiation induces two, possibly more, distinct types of dsb in mammalian DNA, although the nature of these dsb and the mechanisms of their repair has yet to be ascertained. This thesis has by no means answered all the questions required to settle the controversy surrounding the non-denaturing filter elution technique, but it has elucidated some aspects of the assay and has pointed the way for further investigations.

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APPENDIX:

Published article:

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Repair of DNA single-strand and double-strand breaks in the Chinese hamster xrs 5 mutant cell line as determined by DNA unwinding

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Summary

The DNA unwinding technique has been used to measure the induction and repair of DNA strand breaks by X-rays in the X-ray-sensitive (xrs 5) mutant and its parent CHO K1 line of Chinese hamster cells. Results show that frequency of induction of DNA strand breaks was the same for both cell lines. The repair of single-strand breaks was found to be slightly slower in xrs 5 over the first 20 min after X-ray exposure, but the level of repair of ssb reached after an incubation of 1h following X-ray exposure in xrs 5 was the same as in CHO K1. Our results also show that the rate of repair of DNA double-strand breaks in xrs 5 cells was clearly slower than that in CHO K1, supporting the conclusion of Kemp et al. (1984) who used the neutral elution technique, that xrs 5 is defective in the repair pathway of DNA double-strand breaks.

1. Introduction

The DNA double-strand break (dsb) is thought to be a major lesion involved in radiation-induced cell death and other biological end points, e.g. induction of chromosomal aberrations. Failure of cells to repair dsb results in extreme sensitivity to ionising radiation. For example, when irradiated, the yeast line rad 52 is killed by 1–2 dsb per cell (Ho, 1975; Resnick and Martin, 1976). It has been suggested that irradiated mammalian cells may be killed by one or more residual dsb (Blöcher and Pohlit, 1982), however, other evidence points to the induction of chromosomal aberrations as an intermediate step between the initially induced dsb and cell death (Joshi et al., 1982; Bryant, 1984, 1985).

As exemplified in yeast, the study of the relationship between DNA damage and cell death in irradiated cells has been facilitated by the use of genetic mutant lines. In mammalian cells, this type of study has been made possible by the isolation and characterization of mutant X-ray-sensitive (xrs) lines of Chinese hamster cells (Jeggo and Kemp, 1983; Jeggo and Holliday, 1986). The xrs lines 5, 6 and 7 have in addition been shown to exhibit an enhanced chromosomal sensitivity to X-rays (Kemp and Jeggo, 1986; Darroudi and Natarajan, 1987) and to restriction endonuclease

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induced dsb (Bryant et al., 1987). The radiosensitivity of these lines was attributed to a putative defect in dsb repair (Kemp et al., 1984); Single-strand break (ssb) repair was shown to be normal in these lines. Kemp et al. (1984) used the alkaline sucrose sedimentation and alkaline elution techniques to investigate ssb repair, and the results of the two independent assays agreed even though widely different doses were used (200 Gy and 5.7 Gy respectively).

To assay for dsb repair Kemp et al. (1984) used the neutral elution technique of Bradley and Kohn (1979). Neutral elution is a widely used method for the detection of DNA dsb (e.g. Weibezahn and Coquerelle, 1981; Radford, 1985; Sigdestad et al., 1987; Evans et al., 1987) but the results obtained with the method have been controversial as was evident at the recent International Congress of Radiation Research, Edinburgh (1987).

The reasons for this are several. Firstly the results obtained with the neutral elution method are not in agreement with the results of the more established neutral velocity sucrose sedimentation technique. For example, Weibezahn and Coquerelle (1981) obtained a half-time for the repair of dsb of 37 min as compared with the value of 2–4 h as determined by the sedimentation method (Bryant and Blöcher, 1980; Blöcher and Pohlit, 1982). With the sedimentation method, exponential (first-order) dsb repair kinetics were obtained whereas the neutral elution technique showed biphasic kinetics of dsb repair (e.g. Kemp et al., 1984). The kinetics of induction of dsb in cells using neutral sedimentation were found to be linear (Frankenberg-Schwager et al., 1979; Blöcher, 1982) whereas those obtained with neutral elution were shown to be curvilinear (e.g. Radford, 1985). It should also be kept in mind that neutral elution is normally performed at pH 9.6 (Bradley and Kohn, 1979), and that it has recently been suggested that this may reveal other DNA lesions (Tilby et al., 1984). Sigdestad et al. (1987) observed a near linear induction of dsb and exponential dsb repair kinetics when the neutral elution was performed at pH 7.2.

The physical basis of the neutral filter elution method is not yet fully understood, and due to this uncertainty surrounding the method, it was decided to investigate the kinetics of repair in the xrs-5 mutant strain using an alternative method, namely DNA unwinding (Ahnström and Erixon, 1973). Bryant and Blöcher (1980) described how this method could be used to measure both ssb and dsb repair kinetics. The unwinding method does not distinguish between ssb or dsb except on a kinetic basis. When assaying for dsb, the irradiated cells are allowed to repair for 2 h by which time the repair of ssb was found to be complete. The kinetic results at incubation times in excess of 2 h obtained with this technique were in good agreement with results for the kinetics of DNA repair obtained with the neutral sucrose gradient sedimentation method (Bryant and Blöcher, 1980; Blöcher et al., 1983).

This paper describes experiments in which the induction of strand breaks and the repair of ssb and dsb were measured in X-irradiated xrs 5 and CHO K1 cells. Our results show that ssb repair in xrs 5 is similar to that found in normal cells but indicate a deficiency in the repair of dsb in xrs 5. Our results therefore support in principle those of Kemp et al. (1984), although the kinetics of dsb repair measured using DNA unwinding were quite different from those obtained with neutral elution.

2. Materials and methods

Cell culture

The X-ray-sensitive xrs 5 cell line of Jeggo (Jeggo and Kemp, 1983) and its wild-type parent line CHO K1 were used. Experiments were performed with asynchronous populations of exponentially growing cells, which were routinely maintained in Eagle's minimum essential medium (MEM) supplemented with foetal calf serum (15% v/v) and non-essential amino acids.

X-ray exposure

Cells were exposed to X-rays (250 kV with 0.5 mm Cu filtration) at a dose rate of 0.85 Gy/min (survival assays) or 5.4 Gy/min (DNA-unwinding experiments). Dosimetry was checked using a modified Fricke method (Frankenberg, 1969).

Survival assays

Survival assays were carried out on both xrs 5 and CHO K1 in order to verify the reported difference in X-ray sensitivity of the two cell lines. Cells were exposed to X-rays as monolayers in

medium (in air) were appropriately diluted and plated in 5-cm dishes with 5 ml MEM and grown for 6–7 days at 37 °C in 5% CO_2.

DNA unwinding assay

Cells were labelled with 0.1–0.5 μCi/ml of tritiated thymidine (^{3}HTdR) in the presence of 5 μmoles "cold" thymidine for 24 h. Cells were chased in non-radioactive medium for 4–5 h prior to the experiments. This was found to reduce background unwinding in experiments.

The cells were trypsinized and irradiated as a suspension of 2–3 ml in air. In order to obtain dose-effect curves, samples were taken immediately after irradiation (at 0 °C) and pipetted into ice-cold saline solution (0.15 moles/l NaCl) to prevent repair of DNA taking place. For the "short-term" repair (0–1 h) experiments, cell samples in medium at 37 °C were irradiated to a dose of 8 Gy, transferred to a waterbath at 37 °C for various incubation times (4–60 min), and then pipetted into ice-cold saline solution. The procedure of Bryant et al. (1984) of irradiating at various times and collecting samples on ice at a common time point was used. In the case of the "long-term" repair (> 2 h) experiments, cells in suspension were irradiated to a dose of 50 Gy and then returned to petri dishes and incubated at 37 °C and 5% CO_2 for the various times (i.e. 2–8 h). At the appropriate time cells were harvested by trypsinization and added to ice-cold saline solution.

Once in ice-cold saline solution, cells ($2 \cdot 10^6$ cells per sample) were centrifuged to a pellet and the supernatant removed by suction. After vortexing, 1 ml of cold lysing solution (0.03 moles/l NaOH and 1 moles/l NaCl, pH 12) was forcefully added to each sample using a micropipette. Samples were then left undisturbed and under conditions of low-light for 1 h.

Subsequently, samples were neutralised by adding 2.2 ml of 0.02 moles/l NaH_2PO_4 and immediately sonicated for 6–10 sec. After addition of 0.3 ml of 25% sodium dodecyl sulphate, samples were vortexed and then deep frozen (−20 °C) overnight.

Measurement of damage to DNA

The procedure described by Ahnström and Erixon (1973) was followed. Briefly, samples were applied to small affinity columns, containing 0.15 g of hydroxyapatite (Biorad, 50:50 mixture of Bio-Gel HTP and DNA grade Bio-Gel HTP) in plastic syringe bodies, held in a heated aluminium block at 60 °C. Before sample application the hydroxypatite was washed in 2.5 ml of 0.0125 moles/l sodium phosphate buffer (SPB) pH 6.8. After sample application columns were again washed with 0.0125 moles/l SPB. Single-stranded DNA was then eluted with 4.5 ml of 0.125 moles/l SPB and double-stranded DNA was eluted with 4.5 ml of 0.250 moles/l SPB. Samples were then mixed with 0.3 ml 5 moles/l HCl and 5 ml scintillation cocktail (Packard, Instagel) and radioactivity determined by liquid scintillation counting.

The relative mass fraction of DNA in double-stranded form, $(m_{ds}/m_{ds} + m_{ss})$ was obtained from the ratio $A_{ds}/(A_{ds} + A_{ss})$, where A_{ds} is the activity (dpm) measured in the double-stranded fraction and A_{ss} the activity measured in the single-stranded fraction. Dose–effect curves were obtained by plotting the relative mass fraction $m_{ds}/(m_{ds} + m_{ss})$ versus radiation dose. In the repair experiments, the relative mass fraction values were converted to "dose" values by reading dose values off from the dose–effect curves. These dose values expressed in Gy, are indicative of remaining damage following X-ray exposure and were plotted versus incubation time.

3. Results

The survival curves for the two lines are presented in Fig. 1. The reported difference in radiosensitivity and survival curve shape between the CHO-K1 and xrs-5 lines (Jeggo and Kemp, 1983) was confirmed. The CHO K1 line showed a shoulder or threshold region while the xrs 5 line showed exponential killing. D_{10} values were: xrs 5 (1.5 Gy) and CHO K1 (5.7 Gy). These are close to those previously reported by Jeggo and Kemp (1983).

The dose–effect curves obtained with the DNA unwinding method are shown in Fig. 2. The results show that there was no significant difference between the two cell lines in this assay. This data indicates that the same numbers of DNA strand breaks were induced per Gy in xrs 5 as in CHO K1.

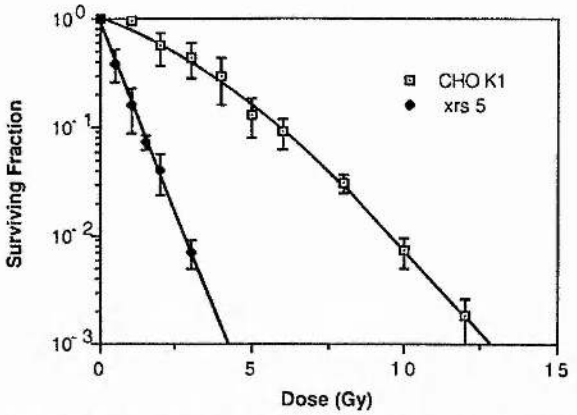

Fig. 1. Survival curves for X-irradiated CHO K1 and xrs 5 cells. Vertical bars represent standard errors of mean values.

Results of short-term repair experiments are given in Figs. 3a and b. The CHO-K1 data is displayed in 3a and the xrs-5 data in 3b. In 3b the dotted line represents the kinetics of CHO K1 from 3a. The data points each represent the mean of 4 individual experiments. Both sets of data (3a and b) show an initial rapid component of repair followed by a levelling off of the curve at between 40 and 60 min after irradiation, indicating that ssb repair was complete. The initial rate of repair found in xrs 5 appeared to be somewhat slower than that in CHO K1 but at later times the

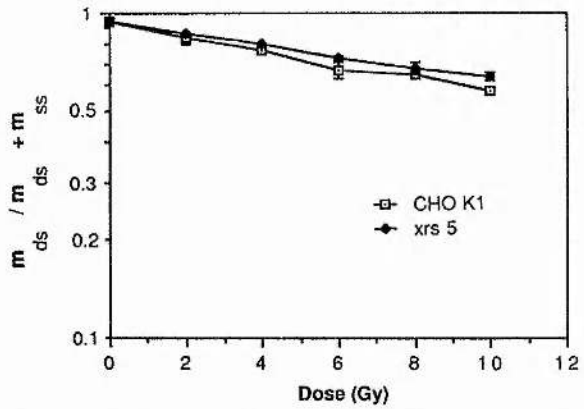

Fig. 2. Dose-effect curves for induction of DNA strand breaks in X-irradiated xrs 5 and CHO K1 measured by the DNA unwinding method. The frequency of strand breaks is inversely related to the logarithm of the function: $m_{ds}/m_{ds} + m_{ss}$. Vertical bars represent standard errors of mean values.

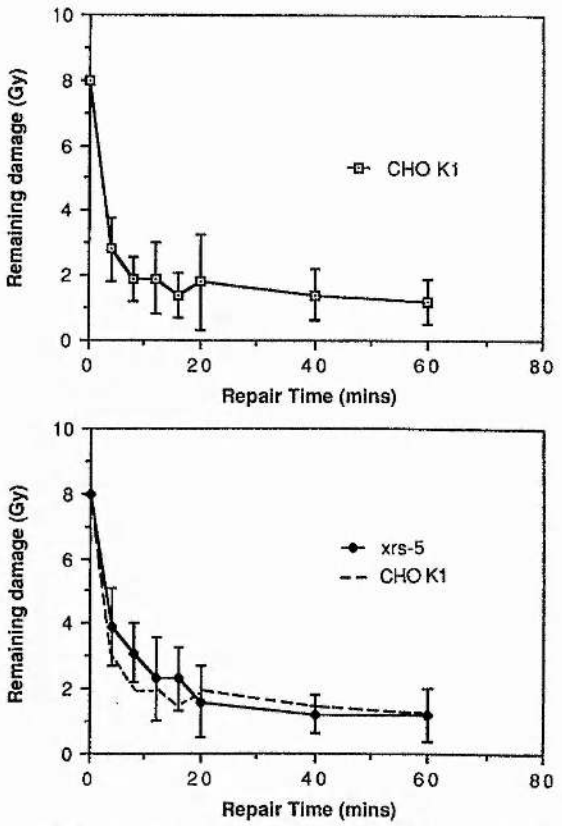

Fig. 3. Kinetics of disappearance of remaining damage (interpreted as repair of single-strand breaks in DNA) as a function of incubation time after X-ray exposure (8 Gy). Panel (a) CHO K1 cells, panel (b) xrs 5 cells. Vertical bars represent standard errors of mean values.

kinetics were similar so that the levels of ssb repair reached after 1 h were the same in the two different lines.

The results of a typical long-term repair experiment are presented in Fig. 4. The results show a clear difference in the kinetics of damage remaining as a function of time in the xrs-5 from that remaining in the CHO K1 line. The remaining damage in CHO K1 disappeared with an overall half-time of about 2 h whereas the kinetics for xrs 5 indicated that the damage decreased by only a small fraction over the 5 h incubation time. We have obtained the same result, namely a clear difference in the two lines with respect to dsb repair in two other independent experiments, al-

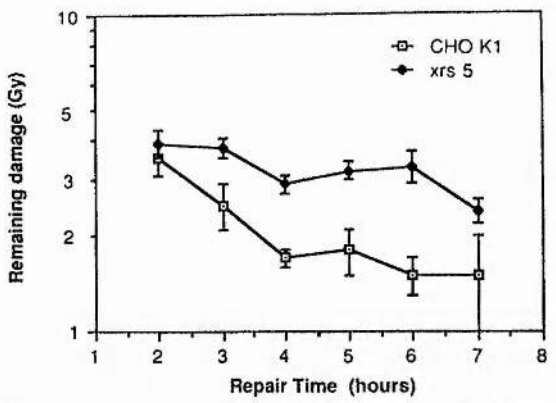

Fig. 4. Kinetics of disappearance of remaining damage (interpreted as repair of DNA double-strand breaks) as a function of time after X-ray exposure (50 Gy) in xrs 5 and CHO K1 cells. Vertical bars represent standard errors of mean values.

though the absolute levels of damage remaining varied from one experimental run to another. For this reason we have not pooled results from this series of experiments.

4. Discussion

Kemp et al. (1984) who used alkaline velocity sedimentation and alkaline elution, reported that in xrs lines 5 and 7 the induction of DNA strand breaks and the repair of ssb was the same as in the parent CHO K1 line. The results of our experiments using DNA unwinding show that induction of DNA strand breaks (comprising both ssb and dsb) is similar in the two lines: xrs 5 and CHO K1 (Fig. 2). Ssb repair (Figs. 3a and 3b) in xrs 5 was slower initially than in CHO K1, but over a period of 1 h the repair in xrs 5 reached the same level as in CHO K1. These results are in essential in agreement with those of Kemp et al. (1984) using alkaline elution. This agreement between the two methods was expected, since Blakely et al. (1983) had previously shown that the alkali unwinding and elution techniques gave comparable relative estimates of radiation induced strand break yields. Using the neutral elution method Kemp et al. (1984) obtained data which suggested that xrs lines were deficient in dsb repair. However the levels of residual dsb (indicated by relative elution values) in each mutant line were not related to the degree of radiosensitivity displayed in survival assays by the same lines.

The neutral elution method has been the subject of considerable controversy recently; the kinetics of dsb induction are usually found to be curvilinear (e.g. Radford, 1985), whereas with the conventional neutral velocity sedimentation technique, linear kinetics of induction of dsb with dose were observed (Lehmann and Stevens, 1977; Blöcher, 1982; Frankenberg-Schwager et al., 1979). In addition, the results of experiments with neutral elution have yielded kinetics of repair of dsb (e.g. Weibezahn and Coquerelle, 1981) which are quite different from those obtained with neutral velocity sedimentation (e.g. Blöcher and Pohlit, 1982; Frankenberg-Schwager et al., 1980). Since there is as yet no sound physical basis for the elution of DNA molecules which are likely to be complexed with proteins of several types (e.g. histones, non-histone proteins and proteins of the nuclear lamina), the kinetics of induction and repair of dsb measured by this method must be subject to considerable uncertainty.

In spite of this uncertainty, the reported defect in dsb repair in xrs mutants (measured by the neutral elution method) does seem to be confirmed by indirect evidence from cytogenetic experiments. Bryant et al. (1987) were able to show that the mutant xrs 5 produces much higher levels of chromosomal aberrations in response to treatment with both blunt-ended and cohesive-ended dsb induced by the restriction endonucleases Pvu II, Eco RV Bam H1 and Eco R1.

In response to the uncertainty surrounding the measurements made with neutral elution we decided to check the kinetics of repair of DNA double-strand breaks in xrs 5 and compare them with those for the parent CHO K1 line using the DNA unwinding method.

The results of these kinetic experiments are shown in Fig. 4. They indicate that the xrs 5 line is deficient in the repair of double-strand breaks in DNA and therefore support the conclusion of Kemp et al. (1984). However the kinetics of repair of dsb in the wild-type parent (CHO K1) found in these DNA unwinding experiments were quite different from those found by Kemp et al. (1984) using the neutral elution method. Although we have not drawn a single straight line through the

points the kinetics shown in Fig. 4 for CHO K1 are not inconsistent with first-order kinetics with a half-time of approximately 2 h. This result is similar to values of $t_{1/2}$ found for repair of dsb in mouse ascites cells measured by neutral velocity sedimentation and DNA unwinding (Bryant and Blöcher, 1980) whereas those of Kemp et al. (1984) indicate a $t_{1/2}$ of approximately 20 min, repair being complete in 2 h.

Another important difference between our results and those of Kemp et al. (1984) is that after 2 h incubation following X-ray exposure our data indicates similar levels of damage in xrs 5 and CHO K1 (Fig. 4) which corresponds to a level of between 3 and 4 Gy of remaining damage whereas that of Kemp et al. indicates a large difference in the percentage of breaks remaining unrejoined after 2 h; after a dose of 90 Gy of γ-rays the parent CHO K1 line had repaired approximately 70% of breaks and the xrs 5 line only 10% of breaks at 2 h following exposure. This serves to emphasize the difference in kinetics obtained with the two methods. In the case of neutral elution, repair of dsb is complete by 2 h following radiation exposure in both cell lines, whereas with the DNA unwinding method a slow decrease in damage, interpreted as repair of dsb, occurs over the period between 2 and 7 h. The repair observed with the unwinding technique after 2 h following irradiation was shown to reflect the repair of dsb as measured by the velocity sedimentation method (Bryant and Blöcher, 1980). The reasons for the difference in kinetics of repair of dsb measured by these methods and that of neutral elution are not yet understood.

Due to uncertainties in determining exact values for $t_{1/2}$ in long-term repair experiments, we have deliberately not tried to quantify the difference in repair kinetics of dsb between xrs 5 and CHO K1 but clearly our results lend strong support to the conclusion of Kemp et al. (1984) that xrs 5 is defective in the repair pathway for DNA dsb.

Acknowledgements

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Neutral filter elution detects only limited inhibition of double-strand break repair by 9-β-D-arabinofuranosyladenine

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Summary

Bradley and Kohn (1979) showed that the neutral filter elution technique detects DNA double-strand breaks (dsb), yet there is still some uncertainty regarding the interpretation of results obtained with this technique (viz. the curvilinear dose-response curve and the rapid repair kinetics). In this report we have investigated the effect of the nucleoside analogue 9-β-D-arabinofuranosyladenine (ara A), a known inhibitor of dsb repair, on the DNA repair in X-irradiated Ehrlich ascites tumour cells as measured by this technique. We have compared the effect of ara A on repair in these cells with results previously obtained with the same cell line and using the neutral velocity sedimentation and DNA unwinding techniques (Blöcher, 1982; Bryant and Blöcher, 1982). Our results suggest that the lesions measured by the neutral elution technique are different from those measured by neutral velocity sedimentation or long-term repair measured by DNA unwinding.

Introduction

The neutral filter elution technique (Bradley and Kohn, 1979) is widely used to measure the induction and repair of DNA double-strand breaks (dsb) in irradiated cells (eg. Weibezahn and Coquerelle, 1981; Radford, 1985), however the validity of the method for measuring dsb is the subject of debate (Radford, 1988). Essentially this debate centres around two observations which differ from those made with neutral velocity sedimentation (eg. Blöcher, 1982; Blöcher and Pohlit, 1982), namely the shape of the dose-response curve for induction of dsb and the kinetics of repair of dsb. Using the filter elution technique, the shape of the dose-response curve is usually curvilinear (Radford, 1985; Iliakis and Okayasu, 1988) whereas that measured by velocity sedimentation is linear (Blöcher, 1982). Several authors have noted that the neutral elution technique detects biphasic dsb repair kinetics in mammalian cells (Woods, 1981; Weibezahn and Coquerelle, 1981 and Sigdestad et al., 1987) whereas neutral velocity sedimentation studies found a single slow component (Lehmann and Stevens, 1977; Blöcher and Pohlit, 1982).

That neutral filter elution can measure dsb in DNA is demonstrated by the increase in relative elution when DNA, released on filters, is treated with restriction endonucleases

(Bradley and Kohn, 1979). However the situation pertaining to X-irradiated cells may be different for a number of reasons. For example X-rays induce a large number of single-strand breaks (ssb) compared to the number of dsb (about 30:1) and it is possible that the ssb (by shearing) may lead to additional dsb during the elution of the DNA through a filter pore (2 μm diameter). Thus, although filter elution may be detecting dsb, this detection may be strongly influenced by the presence (and repair) of ssb (see comment by Ahnström on Radford, 1985).

Wiebezahn and Coquerelle (1981) interpreted the biphasic repair kinetics of neutral elution as discrimination between two types of double-strand break rejoining, namely a fast process involving DNA ligation and a slower component which requires a more complex process, possibly recombination.

In an attempt to resolve the differences in data generated by the two methods we have used an experimental strategy in which we examined the concentration dependence of the inhibition by the nucleoside analogue 9-β-D-arabinofuranosyladenine (ara A) of putative dsb repair measured by the filter elution technique. It has been shown previously using neutral sucrose sedimentation and the unwinding techniques (Bryant and Blöcher, 1982) that ara A strongly inhibits dsb repair and to a lesser extent also ssb repair. In the work reported here we have measured the concentration dependence of inhibition of repair as monitored by both filter elution and DNA unwinding. We have then compared our results with those found previously for inhibition of dsb repair measured by velocity sedimentation (Bryant and Blöcher, 1982).

Materials and methods

Cell culture , radioactive labelling and X-irradiation. Ehrlich ascites tumour (EAT) cells were grown in suspension as previously described (Iliakis and Pohlit,1979). Cells were passaged daily to ensure that the cells remained in exponential growth. Cells at 2.10^5/ml in 40 ml were incubated in 75 cm^2 plastic flasks (Sterilin) for 24 h in the presence of ^{3}HTdR (methyl-^{3}H thymidine, 1.59 TBq/mmol). 74-148 kBq per flask (in 40 ml) was used for the neutral elution technique and 37-74 kBq for the DNA unwinding technique. Equi-molar amounts (1-2 μmol/l) of cold thymidine were added to facilitate uniform uptake of the radioactive thymidine. Cells were X-irradiated (250 kV_p , filtered by 0.5 mm Cu) in suspension in medium at a concentration of 1-2.10^5 cells/ml. The dose-rate as determined by ferrous sulphate dosimetry (Frankenberg,1969) was 5.8 Gy/min. Irradiations were performed at either 0^o C (dose-response experiments) or at 37^o C (repair experiments).

DNA synthesis assay. Unlabelled EAT cells at a concentration of 5- 6.10^5/ml were placed in glass bottles in a water bath and allowed to equilibrate to 37^o C. Ara A was added, in the form of a 10 mmol/l solution in HBSS, to various final concentrations (taken as zero time point). At chosen time intervals, a 1 ml aliquot of cells was removed and 18.5 kBq

^{3}HTdR was rapidly added. After an incubation time of exactly 5 min, 5 ml of cold saline was forcefully pipetted into the sample, which was then put on ice. When all the samples had been accumulated on ice, they were centrifuged, the supernatant medium aspirated and the pellet vortexed. 1 ml of 0.03 M NaOH was then added, followed by 1.5 ml of 0.61 mol/l trichloroacetic acid (TCA) 10 min later. Samples were stored at 4° C overnight to allow full precipitation of DNA.

The DNA was collected onto glass-fibre (Whatman) filters, rinsed twice with ice-cold 0.31 mol/l TCA and washed with ice-cold ethanol. Filters were then dried and after addition of approximately 4 ml of scintillation cocktail (Optiphase MP,LKB) the radioactivity per filter was determined by liquid scintillation counting.

Neutral filter elution. A modified version of the neutral elution technique of Bradley and Kohn (1979) was used. Non-denaturing conditions (pH 9.6) were used throughout. After irradiation samples (5.10^5 cells) were added to ice-cold PBS (phosphate buffered saline) which was then poured into funnels connected to 25mm Millipore 'Swinnex' holders containing 2 μm polycarbonate filters (Nucleopore). The suspension was allowed to run through under gravity, and the cells were rinsed with a further 10 ml of ice-cold PBS. As soon as the filter holders had emptied, they were removed from the funnels and 1 ml of lysis solution (0.025 mol/l Na_2EDTA, 0.1 mol/l glycine, 0.068 mol/l N-laurylsarcosine Na salt) was pipetted into the Swinnex holders. The filter holders were then incubated at 60° C for 1 h (Okayasu and Iliakis, 1989).

After incubation the Swinnex filter holders were reconnected to the funnels and 40 ml of eluting solution (0.02 mol/l EDTA and ~0.06 mol/l tetrapropyl ammonium hydroxide) was poured into the funnels and the elution initiated. A flowrate of 3 ml/h was maintained for an eluting period of 15-18 h. Each fraction (~9 ml collected over 3 h) was mixed with 10 ml Optiphase MP (LKB) scintillation cocktail. Radioactivity per sample was determined by liquid scintillation counting. The polycarbonate filters were removed and thoroughly vortexed in 5 ml Filter Count (Packard) before counting. Internal standards were not used in the experiments described here.

Dose-response. Samples (3 ml of $1\text{-}2.10^5$ cells/ml) were held on ice for ~0.5-1 h prior to irradiation, irradiated on ice and following X-ray exposure returned to ice for ~0.5-1 h. Samples were then mixed with 7 ml ice-cold PBS. The above elution procedure was then followed.

Repair assay. A 10 mmol/l stock solution of 9-β-D-arabinofuranosyladenine (ara A) was made up in HBSS (Hanks buffered salt solution). The solution was acidified with 0.075 mol/l HCl to dissolve the ara A and was added to cell suspensions (in medium) approximately 1 h before irradiation to allow phosphorylation of ara A. Aliquots of 3 ml of cells at a concentration of $1\text{-}2.10^5$ /ml were put into 5 ml plastic bottles (bijou), which were gassed and held at 37° C in a water-bath throughout the assay. After irradiation (30 Gy), the

cell suspensions were returned to the water-bath for various repair times. The irradiations were staggered such that all samples could be collected at a common time point. To halt repair, samples were poured into a large volume (7 ml) of ice- cold PBS and further processed as described above.

DNA unwinding assay. The procedure followed was described previously for CHO K1 cells (Costa and Bryant 1988) under the heading 'long term repair'. The only difference was that in the present work EAT cells were used, which did not require trypsinization.

Results

The results of the DNA synthesis (pulse-labelling) assay are given in Fig. 1. The incorporation of ^{3}HTdR into DNA during a 5 min pulse was found to be constant over a 1 h period of incubation of cells in medium at 37° C. When ara A was added either at 10 or 100 µmol/l, a strong decrease in incorporation was observed which increased with time. An ara A concentration of 100 µmol/l was found to be sufficient to almost completely inhibit the incorporation of ^{3}HTdR. This result implies that semi-conservative DNA synthesis can be completely inhibited at 100 µmol/l ara A.

The results of the DNA unwinding assay are presented in figure 2. The remaining damage following irradiation and incubation for various times (expressed in Gy) was estimated from the dose-response curve (data not shown). In control (X-irradiated) samples, remaining damage disappeared with time approximately to first-order kinetics, as has been found previously for EAT cells (Bryant and Blöcher, 1980). This slow disappearance of damage ($t_{1/2}$ =2-3 h) corresponded to the kinetics measured by neutral sucrose sedimentation and was therefore interpreted as repair of dsb. The results show that an almost complete inhibition of dsb repair occurs in the presence of 400 µmol/l ara A. The data at 1600 µmol/l ara A also show no repair, but reveals a higher level of remaining damage than was observed at 400 µmol/l.

The dose-response curves for induction of damage measured by neutral filter elution at pH 9.6 are given in Fig. 3. Background elution values, obtained from unirradiated samples, have been subtracted). The data shown in Fig.3 does not unequivocally represent a sigmoidal fitting, but the curve is based on detailed dose response studies done previously. Doses were limited to 30 Gy in the repair experiments so that they remained within the linear portion of the dose-response curve. Fig. 3 shows that the presence of ara A did not affect the amount of damage induced by X-rays. Similar results were obtained with the DNA unwinding technique (data not shown).

The results of the repair assay using neutral filter elution are given in Fig. 4a and Fig. 4b. The data suggest biphasic repair kinetics and estimates of the half times of the rapid and slow repair components for the control samples were 6 min and 114 min respectively.

Figure 4b shows the data calculated as the percentage of breaks (assumed to be dsb) rejoined with time. These values were obtained using the following formula:

$(1-x_t/x_0)100$

where x_t = the fraction of radioactivity eluted after repair time t and

x_0 = the fraction of radioactivity eluted for the unrepaired sample (background elution values have been subtracted in both cases).

The results show that for control samples approximately 90% of the breaks were rejoined during the 180 min period following irradiation. 75% of the lesions were repaired in the presence of 400 µmol/l ara A and 60% in the presence of 1600 µmol/l ara A.

Discussion

The modifications to the neutral elution technique of Bradley and Kohn (1979) suggested by Okayasu and Iliakis (1989) were implemented in order to increase the sensitivity of the method without significantly altering the pattern of elution. Okayasu and Iliakis (1989) postulated that the strong treatment with detergents and elevated lysis temperature disrupts the DNA-protein complex and reveals DNA lesions that would otherwise be obscured, hence the increase in the sensitivity of the assay. Using these modifications we found essentially the same induction and repair results as have other authors (Iliakis et al. 1988; Kemp et al. 1984), which indicates that the type of lesion being measured has not changed. Futhermore the repair kinetics we obtained using TPAH (tetrapropyl ammonium hydroxide) in the eluting solution (see control data in Fig. 4) are almost identical to the tris solution results of Koval and Kazmar (1988), unlike their own results.

It was shown previously that repair of dsb was fully inhibited in EAT cells by ara A at a concentration of 400 µmol/l (Bryant and Blöcher, 1982). This result was obtained with both the neutral velocity sedimentation technique and with the DNA unwinding assay where repair was followed at times between 2 and 7 h after exposure to 100 Gy. In the present study we have observed the effect of ara A on the repair of dsb as measured by neutral filter elution and the DNA unwinding technique. The latter technique was used solely to verify the results of Bryant and Blöcher (1982) and we were able to confirm essentially a full inhibition of repair of dsb in EAT cells by ara A at a concentration of 400 µmol/l (Fig. 2). At 1600 µmol/l full inhibition of repair of dsb was also observed, however the line was displaced upwards indicating a higher level of residual damage. It is possible that this results from inhibition of ssb repair which was also observed in previous experiments at concentrations of ara A in excess of 500 µmol/l (Bryant and Blöcher, 1982), but the effect of complete deregulation of cell metabolism at this high ara A concentration cannot be ruled out.

The data obtained with neutral filter elution (figures 4a and 4b) shows that more than 80% repair occurred (when normalised to control value of 90%) in the presence of 400 µmol/l ara A,. About 67% repair occurred when compared with controls at 1600 µmol/l ara A. These are in strong contrast to those found with the neutral velocity sedimentation and slow component of DNA unwinding techniques where a complete inhibition was observed at 400 µmol/l ara A.

Due to the different experimental conditions used, it was not possible to draw a direct comparison between the frequencies of lesions measured with the two above mentioned techniques. The aim of this study was rather to compare the rates of repair in the presence of ara A and to determine if the neutral elution technique, thought to detect DNA dsb, revealed the same extent of inhibition of dsb repair. Our results with ara A therefore suggest that the neutral filter elution technique measures a lesion different from that measured by neutral sucrose centrifugation and DNA unwinding. The fact that only limited inhibition of dsb repair was observed in the presence of ara A indicates that neutral filter elution detects a specific type of double-strand break which is repaired under conditions where DNA polymerization is drastically reduced.

Kemp et al. (1984) noted that 50% of the DNA strand breaks, as measured by neutral elution, had disappeared within ~40 minutes after exposure to X-rays. This $t_{1/2}$ value is at variance with that measured by velocity sedimentation, which is some 2-4 h depending on conditions (Bryant and Blöcher, 1980; Blöcher and Pohlit, 1982). If we assume biphasic repair kinetics for the neutral elution technique, as have several other authors (Woods, 1981; Weibezahn and Coquerelle, 1981 and Sigdestad et al., 1987), the half-times of the initial and slow components are 6 and 114 minutes respectively. The half-time of the slow component is not dissimilar to the half-time of dsb repair ($t_{1/2}$ = 2-4 h) measured by velocity sedimentation (Bryant and Blöcher, 1980). This is in agreement with Weibezahn and Coquerelle (1981) and Woods (1981), who noted the rapid dsb repair of neutral elution was similar to the kinetics of ssb repair, however proposed that the rapid component represents a ligation process. They proposed that the slow dsb repair may require a more complex process, possibly recombination.

Mirzayans et al. (1988) suggested that the type of lesion induced determines the repair enzyme that is activated. If ara A inhibits only one of these repair enzymes (eg. a DNA polymerase), it is conceivable that the lesions detected by the neutral elution technique do not require the repair enzymes that are inhibited by ara A. This, together with the observation of the two components of dsb repair, would support the argument for the existence of at least two different types of dsb.

In conclusion, from the response of cells to ara A our data suggest the existence of more than one type of dsb and that the lesions measured by the neutral elution technique

are different from those measured by neutral velocity sedimentation or long-term repair measured by DNA unwinding technique.

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Figures:

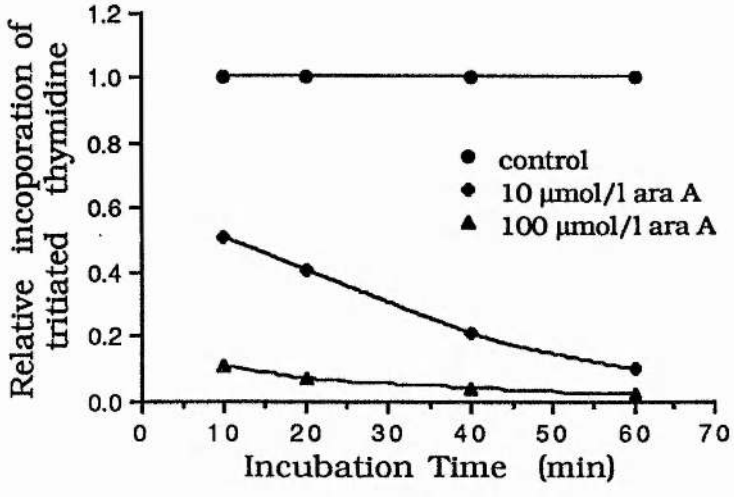

Fig. 1. DNA synthesis assay. Relative incorporation of tritiated thymidine into the DNA of Ehrlich ascites tumour cells incubated at 37° C in the presence and absence of ara A.

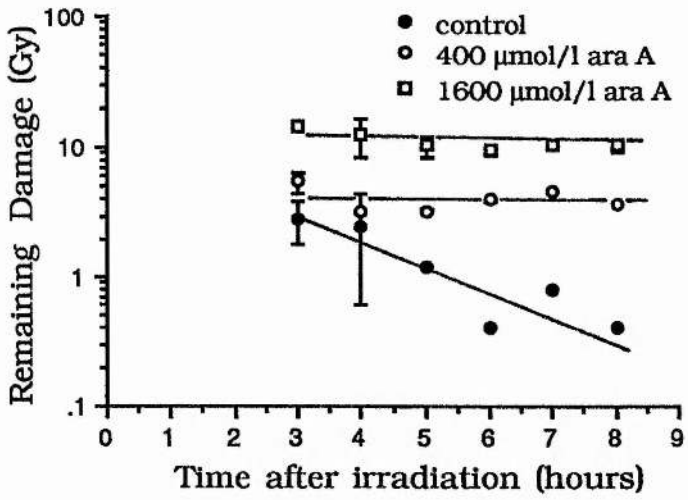

Fig. 2. Kinetics of disappearance of remaining damage (interpreted as repair of DNA double-strand breaks) as a function of time after X-ray exposure (50 Gy) in the presence and absence of ara A. Vertical bars represent standard errors of mean.

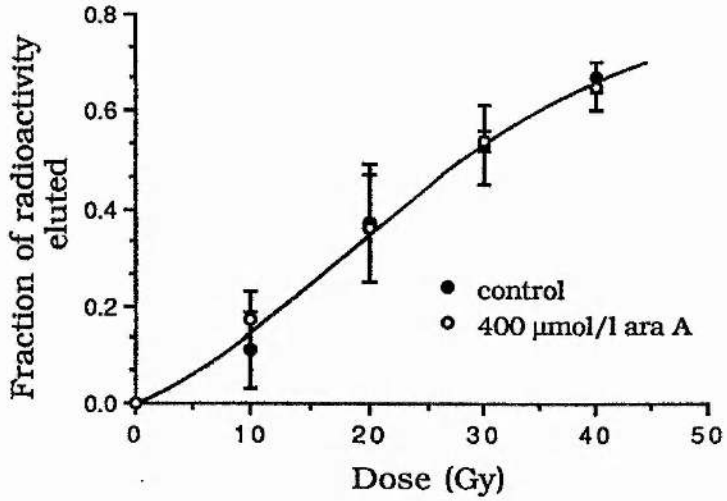

Fig. 3. Dose-response curve for Ehrlich ascites tumour cells, in the presence and absence of ara A, as measured by the neutral filter elution technique (pH 9.6). The fraction of DNA eluted at 16 h was used and the points represent the mean of four experiments and the vertical bars represent standard errors of mean values.

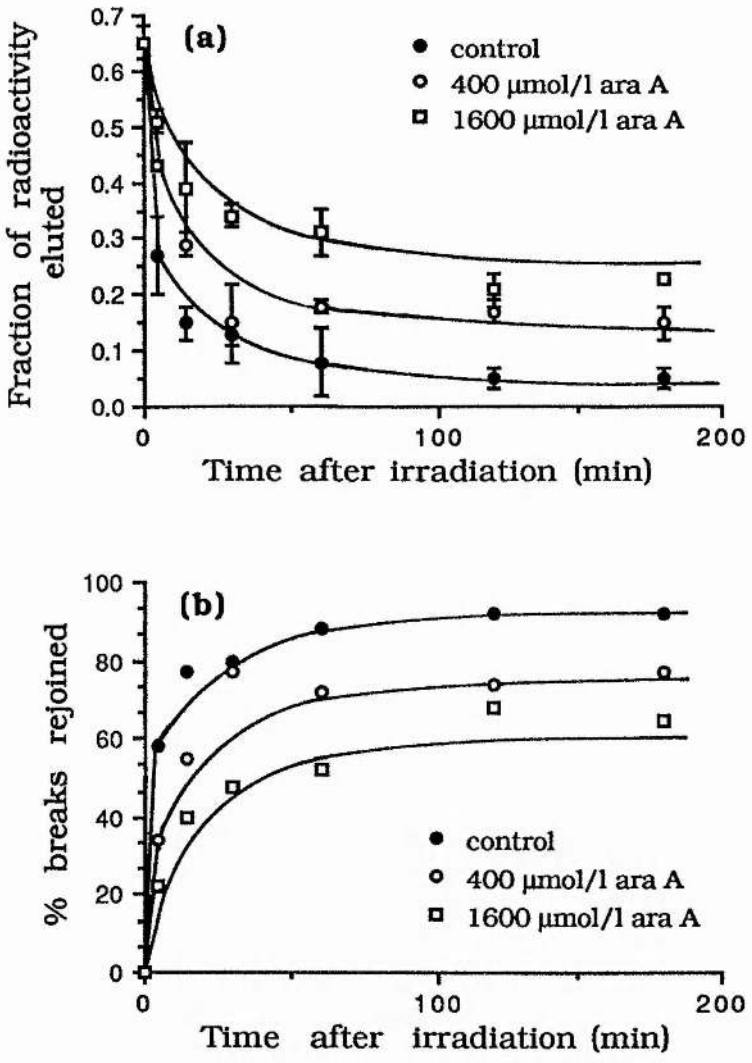

Fig. 4. (a) Kinetics of disappearance of DNA strand breaks as measured by neutral filter elution (pH 9.6), as a function of incubation time after X-ray exposure (30 Gy), in the presence and absence of ara A. The points represent the mean of three experiments and the vertical bars the standard errors of mean (b) Data from panel (a) calculated as percentage breaks rejoined (see text).

The induction of DNA double-strand breaks in CHO cells by *Pvu* II: kinetics using neutral filter elution (pH 9.6).

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Abstract

Chinese hamster CHO K1 cells were treated with the restriction endonuclease *Pvu* II during electroporation and assayed for DNA double-strand breaks (dsb). Dsb were measured by the non-denaturing filter elution technique (pH 9.6) at various times up to 24 h after restriction endonuclease (RE) treatment. The frequency of dsb following electroporation in the presence of 200 units/ml *Pvu* II increased over the post-treatment incubation period. This was found not to be due to cell or DNA degradation indicating that *Pvu* II remains active for at least 24 h inside the cell. We suggest that these kinetics of dsb result from a competition between incision (by *Pvu* II) and dsb repair.

1. Introduction

Cell killing after exposure to ionising radiation has been attributed to unrepaired or misrepaired DNA double strand breaks (dsb) e.g. Blöcher and Pohlit 1982, Frankenberg *et al.* 1984, Curtis *et al.* 1987. For this reason much attention has been directed towards the measurement of the the induction and repair kinetics of dsb, especially in mammalian cells. The role of dsb in the cytogenetic and cellular effects of radiation has recently been made clearer as a result of experiments using restriction endonucleases (RE) to mimic the effects of radiation (Bryant 1984; 1985, Bryant *et al.* 1987). Much work has subsequently been done to investigate the clastogenic effects of RE however little information is available as to the induction of dsb (Bryant 1984, Natarajan *et al.* 1985) and nothing is known about the kinetics of repair of dsb (see Bryant 1988 for review). Recently the use of electroporation to 'porate' cells (Winegar *et al.* 1989) has improved studies of chromosomal aberrations and, as we report here, enabled a study to be made of the induction of dsb. The principle of the cell electroporation technique is that the voltage gradient induced causes the plasma membrane to break down in localised areas, forming pores (Knight 1981) the size and lifetime of which are sufficient to allow the uptake of large molecules up to the size of DNA (Chu *et al.* 1987).

In this study we have used electroporation to introduce *Pvu* II into Chinese hamster ovary cells and the non-denaturing filter elution technique (pH 9.6) of Bradley and Kohn

(1979) to follow the induction and repair of dsb. *Pvu* II was chosen since our previous studies (Bryant 1984, 1985) have shown that this enzyme causes high levels of chromosomal aberrations and cell killing in rodent cells.

2. Materials and methods

2.1 *Cell culture and labelling*

Asynchronous populations of exponentially growing Chinese hamster ovary (CHO K1) cells were used. The cells were routinely maintained in Eagle's minimal essential medium (MEM) supplemented with 10 % v/v calf serum (to which had been added 100 µmol/l $FeCl_3$). 75 cm^2 plastic tissue culture flasks (Sterilin) were seeded with 1.10^6 cells and labelled with 3.7 kBq/ml (1.59 TBq/mmol) tritiated thymidine for 48 h.

2.2 *Purification of Pvu II and electroporation treatment*

Pvu II was purified using an Amicon 10 ultrafilter (Bryant and Christie 1989) to remove storage buffer and was then diluted to 10 units *Pvu* II/µl in calcium free Hanks balanced salts solution (HBSS) containing 6 mmol/l $MgCl_2$ and 1 % BSA (bovine serum albumen) .

After trypsinization, cells were suspended in MEM to give a cell concentration of 1.10^6 cells/ml. Electroporation in MEM was performed using a BRL Cell-Porator. 20 µl (200 units) of purified *Pvu* II was mixed with 1 ml of cell suspension in an Eppendorf tube before being pipetted into a disposable electroporation chamber. The electroporation conditions of Winegar *et al.* (1989) were used: field strength 650 V/cm, capacitance 1600 µF and with the electroporator set at low resistance. Throughout treatment samples were held at ambient temperature. Immediately after electroporation samples were poured into V-tubes and the chamber rinsed with 3 ml MEM. Samples were centrifuged and the supernatant aspirated. After resuspension in 4 ml MEM, samples were poured into tissue culture petri dishes, which were then placed in a humidified, 5 % CO_2 incubator at 37° C for various incubation times. The treatments were staggered such that all samples were collected at a common time point.

2.3 *Assay of DNA double-strand breaks*

After removal of the medium, cells were trypsinised from dishes, resuspended in ice-cold PBS (phosphate buffered saline) and loaded on to 2 µm polycarbonate filters (Nucleopore) for the non-denaturing filter elution. The method of Bradley and Kohn (1979) was followed (pH 9.6) with modifications as described by Okayasu and Iliakis (1989). Cells were lysed in 1 ml of 0.025 mol/l Na_2EDTA, 0.1 mol/l glycine and 0.068 mol/l N-lauroylsarcosine (Na salt), at 60° C for 1 h. The eluting solution; 0.02 mol/l EDTA and ~0.06 mol/l tetrapropyl ammonium hydroxide (20 % in water) was pumped at 3 ml/h and 3 hourly fractions were collected over 15-18 h.

After addition of scintillation cocktail (Optiphase MP, LKB) the radioactivity per fraction was determined by liquid scintillation counting. Filters were counted after being thoroughly vortexed in 5 ml Filter Count (Packard).

2.4 *Assay of DNA precipitability*

Cells were labelled as described above. Samples of 1.10^6 cells were taken at 7, 12 and 24 h post-treatment incubation, washed by centrifugation in HBSS to remove medium, lysed for 10 min in 0.03 mol/l NaOH and DNA precipitated with trichloracetic acid (TCA) to a final concentration of 0.31 mol/l and held on ice overnight. Samples were filtered on to glass fibre filters (Whatman) and washed with 0.31 mol/l TCA and ethanol. Both filters and filtrate were counted for radioactivity using Optiphase MP (LKB).

3. Results and discussion

The elution profiles for *Pvu* II treated cells were found to have a similar shape to those of previous experiments with X-rays giving similar levels of elution (Figure 1), which suggests that the eluted DNA fragment size distributions are similar to those for X-rays. The fraction of radioactivity eluted is assumed to reflect the number of dsb present in the DNA. The frequency of dsb in *Pvu* II treated cells (Figure 2) appeared to increase initially quite rapidly, followed by a more gradual rise from 8-24 h. Background elution values (untreated samples) have been subtracted in both sets of data. Despite the larger error values at longer incubation times it is evident that the numbers of dsb are still increasing over the 24 h post-treatment. The frequency of dsb induced by 200 units *Pvu* II at 24 h was approximately equivalent to the dsb induced by 20 Gy of X-rays. The control values i.e. the number of dsb due to electroporation only, are comparatively small and remain more or less constant over the 24 h time period. These experiments demonstrate the induction of dsb in *Pvu* II treated cells, and explain the lack of success in preliminary experiments, in which cells were lysed for analysis 0.5-1 h after treatment, to detect dsb by non-denaturing filter elution in electroporated cells treated with RE.

The possibility that the results in figure 2 (increasing elution with time) reflect DNA degradation at the extended incubation times rather than cutting of specific *Pvu* II recognition sites, was tested using two assays on cells at 7,12 and 24 h post-treatment. Using the trypan blue dye exclusion assay it was found that less than 0.5% of the cell population showed up positive (blue; indicating death) in all samples i.e. both electroporated controls and enzyme treated samples. In an assay of DNA precipitability using tritiated thymidine labelled cells we showed that only a very small and constant fraction of DNA (~1% probably representing unbound nucleotides) failed to precipitate in TCA (Table I) eliminating the possibility that DNA degradation had occurred in either electroporated controls or enzyme treated cells. We therefore conclude that the increase in

elution of DNA from *Pvu* II treated cells over the 24 h post-treatment incubation period was the result of a progressive accumulation of dsb induced by *Pvu* II. It seems surprising that *Pvu* II remains active inside the cell for a period of 24 h. We have no explanation for this, however we have demonstrated that this enzyme (in a purified form) is extremely stable in *vitro*, retaining its full activity at 37 ^{o}C for 24 h in HBSS/BSA, as measured by a cutting assay using the plasmid pBR322 (data not shown).

The significant finding of the experiment shown in figure 2 is that the kinetics of induction of dsb show that the number of dsb are still increasing, even at 24 h post-treatment. If we assume that the repair of dsb is occurring simultaneously with incision of DNA by Pvu II, the results imply that the *Pvu* II is cutting the DNA at a higher rate than the repair can take place. If this were true it could mean that at lower enzyme doses the kinetics of accumulation of breaks could be substantially different, for example a transient increase in dsb might occur followed by a decrease due to repair. At this stage the sensitivity of dsb assays precludes measurement at much lower enzyme doses. It is however also possible that the curve represents the cutting of *Pvu* II sites alone, i.e. without repair; and at this stage we cannot differentiate between these two hypotheses.

With respect to the gradual increase in dsb with time, the action of RE at this concentration appears to differ significantly from that of a single acute dose of ionising radiation, which induces prompt damage, although the RE treatment might be analogous to irradiation at low dose-rate where dsb are also induced over a long time period. If this kinetic applies also to low RE doses this must be taken into account especially when interpreting the results of experiments in which RE are used to mimic the cytogenetic effects of ionising radiation, since the sampling times that are generally chosen lie within the 5-24 h post-treatment time interval.

Our data for the induction of dsb by *Pvu* II (Fig. 2) appears to conflict with cytogenetic data obtained at lower enzyme doses in that cells harvested after long periods of incubation show largely chromosome aberrations (Obe and Winkel 1985; Winegar and Preston 1988), whereas if the DNA were subjected to continuing breakage one might predict that cells harvested at long intervals after treatment would always show a mixture of chromosome and chromatid aberrations. The only possible explanation we can offer at this time is that, as suggested above, the cutting and repair of cellular DNA may at the low RE doses (generally employed in cytogenetic studies) result in only a transitory increase in dsb at relatively short times after treatment which could give rise to cells containing largely chromosome-type aberrations.

The increasing frequency of dsb over 24 h may thus indicate that the competition between enzymatic incision by *Pvu* II and repair of the induced dsb is tipped in favour of incision, at the enzyme concentration (200 units/ml) we have used. Experiments using other RE are currently underway in our laboratories.

Acknowledgement

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Figures:

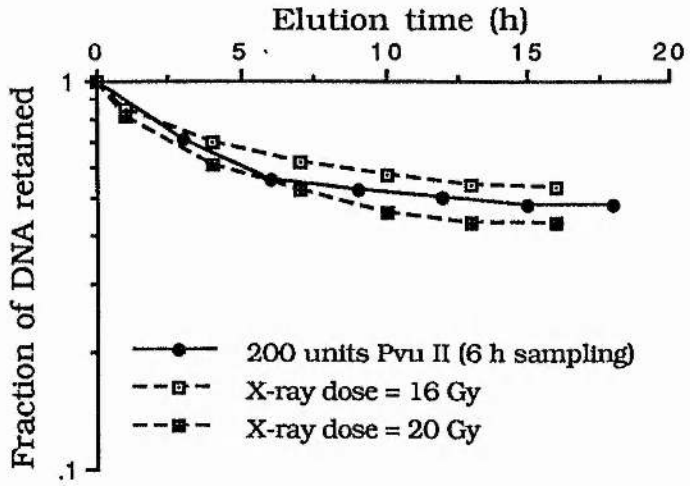

Figure 1. The elution profiles of X-irradiated and *Pvu* II treated CHO cells using the non-denaturing filter elution technique at pH 9.6. X-ray doses were chosen to give similar levels of elution after 16 h to that of electroporated cells treated with 200 units/ml *Pvu* II.

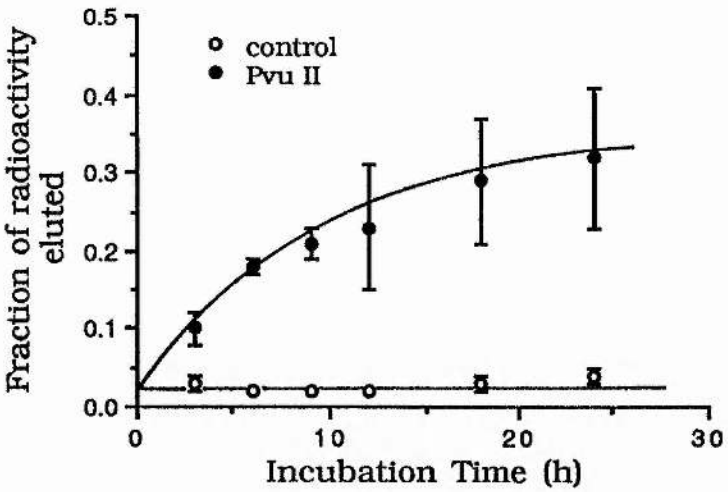

Figure 2. Induction of DNA double-strand breaks in electroporated CHO cells treated with Pvu II (200 units/ml), as measured by neutral elution, up to 24 h post-treatment. This data represent the mean and standard errors of mean values of 2 experiments.

Table I. Radioactivity in the filtrate of trichloracetic acid (TCA) precipitated DNA of electroporated cells treated with or without the restriction endonuclease *Pvu* II and incubated in medium for various times.

Treatment	**Incubation time (h) at 37 °C**	**% of radioactivity in filtrate**
Electroporated controls	7	1.04
	12	1.40
	24	1.31
Electroporated in presence of 200 units/ml *Pvu* II	7	1.36
	12	1.32
	24	1.10